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ADENOSINETRIPHOSPHATASE STUDY DURING RAT LIVER DAMAGE

I. ATP-ASE ACTIVITY OF RAT LIVER DURING REGENERATION AFTER PARTIAL HEPATECTOMY[1]

BY CLAUDE ALLARD[2] AND ANTONIO CANTERO[2]

Abstract

Adenosinetriphosphatase activity of the adult albino rat liver varies during stimulated growth caused by partial hepatectomy. Effectively the mitochondrion loses 48% of ATP-ase activity during the first day of regeneration and begins to recuperate ATP-ase activity only after the eighth day of regeneration. Most probably the microsomes (small granules) or the soluble part of the cell are the site of ATP-ase activity fluctuation during rat liver regeneration. The nuclei ATP-ase activity is relatively constant during regeneration. A low ATP-ase activity in the mitochondrion and supernatant fraction of the cell corresponds to a high rate of cell division. The ATP-ase activity of the regenerating liver is different from the ATP-ase activity of the liver tumor.

Introduction

In recent papers published by a group in this laboratory the variations of the rat liver nucleodepolymerases activity were indicated during carcinogenesis (6) as well as during regeneration (7). In the long-range biochemical investigation of the rat liver during general damage, it has appeared interesting to study adenosinetriphosphatase (ATP-ase) in a manner similar to that used in the study of the nucleodepolymerases.

In the normal rat liver cell, approximately fifty per cent of the specific ATP-ase activity is known to be concentrated in the isolated mitochondrial fraction (11, 13). The liver tumor mitochondrial fraction contains approximately four times less specific ATP-ase activity (13). The decrease in ATP-ase activity of the mitochondrial fraction of the liver tumor is compensated by a corresponding increase in activity in the microsome or soluble portion of the cell, and the total activity of the tumor homogenate is not different from the activity of the normal liver tissue. It is of interest to know whether the same phenomenon occurs in the rat liver cells during stimulated growth process of the liver, after partial hepatectomy.

[1] *Manuscript received March 7, 1952.*

Contribution from Montreal Cancer Institute, Research Laboratories, Notre-Dame Hospital, Montreal, Canada. This investigation is being supported by a grant from the National Cancer Institute of Canada.

[2] *Montreal Cancer Institute.*

The mitochondrion as a unit of measurement is employed in the present study of the mitochondrial fraction ATP-ase activity. We have stressed elsewhere (2, 3) that the mitochondrion seems more appropriate than the cell or other basis in the study of the mitochondrial fraction properties.

Material and Method

A total of 90 albino adult rats of different sexes weighing from 120 to 220 gm. are partially hepatectomized (66%) according to the method of Higgins and Anderson (10). Before and after the operation the animals are fed Purina Fox Chow and water *ad libitum*. As previously described (2) 1, 2, 3, 4, 6, 8, 12, and 20 days after partial removal of the liver, five animals or more are killed, the liver excised, homogenized in 0.25 *M* sucrose (4), and fractionated to isolate cellular particulates. Five hours after the animal is killed, the homogenate and the separate nuclear (N), mitochondrial (M), and the supernatant (S) fractions are assayed for ATP-ase activity according to the method of DuBois and Potter (9) in the presence of optimal calcium chloride (10^{-3} *M*). The sodium salt of ATP, sold by Schwarz Laboratories, Inc. New York, is employed.

The total number of cells per gram of fresh regenerating liver reported by Price and Laird (12) are used to express ATP-ase activity per cell, as suggested by Boivin and the Vendrelys' (5) and others (8, 11). The total number of mitochondria per gram of fresh regenerating liver determined recently in this laboratory (3) is utilized to evaluate the ATP-ase activity per mitochondrion.

Results

Fig. 1 shows the mean total rat liver homogenate specific ATP-ase activity on a wet weight and on a per cell basis during regeneration after partial hepatectomy. The number of animals used is given in Table I. The activity per cell is obtained by dividing the ATP-ase activity per gram of tissue by the number of cells per gram of fresh regenerating liver as given by previous investigators (12). ATP-ase activity of the whole homogenate diminishes abruptly during the first day after partial removal of the liver. During the second and third day, the ATP-ase reaches its lowest value of 4170 units per gram of fresh tissue or 30.5×10^{-6} units per cell. These values correspond to about 50% of normal ATP-ase activity. A progressive increment in ATP-ase activity, beginning during the fourth day, is followed by a decline in activity detectable during the sixth and eighth day. The ATP-ase activity of the regenerating liver homogenate tends to reach a normal value after 12 days of regeneration.

Fig. 2 shows the constancy of the corrected[1] ATP-ase activity of the nuclear fraction on a per gram of original tissue and per cell basis.

Fig. 3 shows the corrected ATP-ase activity of the mitochondrial fraction on a per gram of original tissue, per cell, and on a per mitochondrion basis.

[1] *The ATP-ase activity of the (N) and (M) fraction in Fig. 2 are corrected for the number of mitochondria contaminating the nuclear fraction (3).*

On either basis a rapid drop in ATP-ase activity is evident during the first day of regeneration. The loss on a per gram basis is 63%; on a per cell basis: 50% and on a per mitochondrion: 48%. On a per gram and per cell basis the

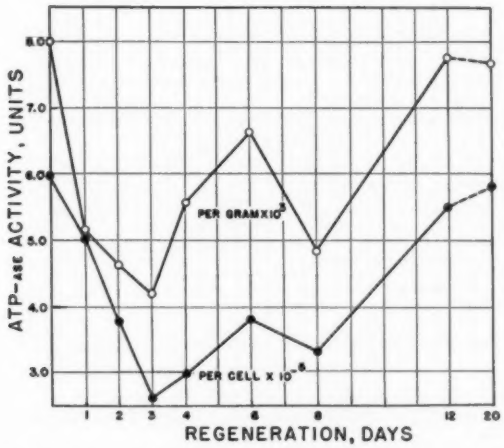

FIG. 1. ATP-ase activity of rat liver homogenate during regeneration. The activities are expressed as micrograms phosphorus liberated in 15 min. by the equivalent of 1 gm. of fresh tissue or by one average liver cell.

activity still decreases during the second and third day of regeneration. After the third day the ATP-ase activity per gram and per cell increases progressively to attain a normal value after 20 days of regeneration. On a mitochondrion

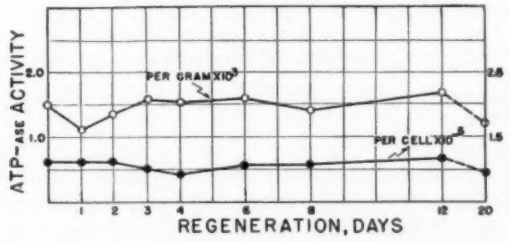

FIG. 2. ATP-ase activity of the nuclear fraction of the regenerating rat liver. Same unit as in Fig. 1.

basis, except during the first day, the phenomenon is different. The ATP-ase activity per mitochondrion is constant from the second to the eighth day, when the enzyme activity progressively returns to a normal value.

Fig. 4 shows the ATP-ase activity in the supernatant fraction. The two curves are quite similar, except during the first and sixth day where slight differences exist.

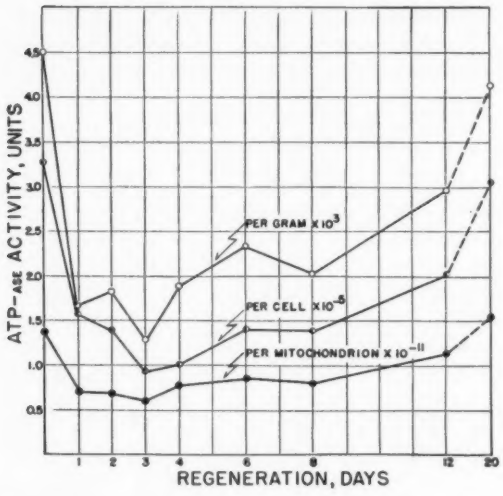

FIG. 3. ATP-ase activity of the mitochondrial fraction of the regenerating rat liver. Same unit as in Fig. 1.

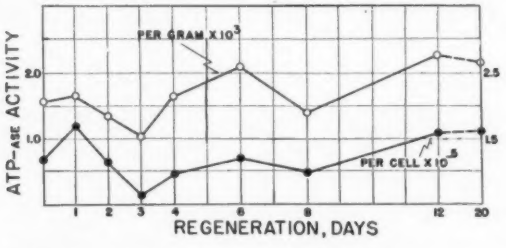

FIG. 4. ATP-ase activity of the supernatant fraction of the regenerating rat liver. Same unit as in Fig. 1.

Table I shows the number of animals used and the mean percentage distribution of ATP-ase activity in the corrected (N), (M), and (S) fractions during regeneration of the rat liver. The percentage distribution is calculated by dividing the ATP-ase activity per gram-equivalent of the (N), (M), and (S) fractions by the original homogenate total activity. The recovery of ATP-ase homogenate activity in the isolated fractions is generally about 90–100%. Eight recoveries were below 80% and are not included in the results. They were assumed false because of technical errors. But recently Novikoff *et al.* have shown that the lack of recovery is due to the inability of calcium to activate, in some instance, the mitochondrial fraction ATP-ase. When magnesium is added to the incubation medium, the recovery has been shown by these authors to be always near 100% (11). Swanson on the other hand (14),

TABLE I

MEAN CORRECTED PER CENT DISTRIBUTION OF ATP-ASE ACTIVITY IN THE (M), (N), AND (S) FRACTIONS DURING RAT LIVER REGENERATION

Day	Number of animals	(N) Fraction corrected	(M) Fraction corrected	Fraction (S)	Recovery
0	11	18.7	56.1	19.4	94.2
1	7	21.5	39.7	32.6	93.8
2	7	28.8	37.4	30.4	96.6
3	6	37.6	32.3	25.0	94.9
4	6	27.9	35.1	30.6	93.6
6	6	24.3	36.5	31.8	92.6
8	6	29.4	41.7	29.6	100.7
12	5	22.2	38.1	29.6	89.9
20	5	15.7	54.8	28.5	99

has suggested the use of separate assay systems with calcium or magnesium for the study of the different ATP-ases (14).

Table II summarizes the statistical analysis of the results. The t test has been applied between critical points of the curves plotted in Figs. 2, 3, and 4. Statistical analysis of the results on a per cell basis is impossible because the mean number of cells reported by previous investigators (12) are used.

TABLE II

RESULTS OF THE APPLICATION OF THE t TEST BETWEEN CRITICAL POINTS OF THE CURVES PLOTTED IN FIGS. 2, 3, AND 4

Fraction	Basis	Day:	Significant*	Not significant*
(N)	Wet weight	0 Versus 1		+
		0 " 4		+
		1 " 4		+
(M)	Wet weight	0 " 1	+	
		0 " 4	+	
		1 " 3	+	
		1 " 4		+
(M)	Mitochondrion	0 " 1	+	
		0 " 4	+	
		1 " 4		+
		3 " 4		+
		1 " 6		+
(S)	Wet weight	0 " 1		+
		0 " 3	+	
		0 " 6	+	
		0 " 8		+

* *Significance is given with a probability of 0.95.*

Discussion

The whole rat liver homogenate ATP-ase activity varies greatly when this organ is damaged by partial hepatectomy. At a same period of high rate of liver cell division (1, 12), corresponds a low ATP-ase activity in the average liver cell. The gross variations in ATP-ase activity measured on total homogenate are an algebraic sum of ATP-ase activity fluctuations in the separate cell particulates and of a change in the number of mitochondria. A close scrutiny of the changes occurring in the cell particulates clearly confirms the phenomenon. Furthermore the use of the mitochondrion as a unit of measurement for the ATP-ase activity of the mitochondrial fraction eliminates the fluctuations in the mitochondrial population as a cause of enzyme variation during liver regeneration.

Evidently the liver nuclei ATP-ase activity is not affected during regeneration of the rat liver. On the other hand, the mitochondrial and supernatant portion of the cell would seem to lose as well as regain ATP-ase activity during regeneration. The percentage distribution of ATP-ase activity in the (M) and (S) fraction illustrates the fact that the variations occurring in these fractions are nearly parallel to those measured on total homogenate since the percentage distribution is relatively constant throughout the major part of the period of regeneration studied. However the (M) fraction ATP-ase activity on a mitochondrion basis shows a decrease only during the first day and thereafter remains constant to the eighth day. The variations in ATP-ase activity of the mitochondrial fraction when expressed on a per gram or per cell basis would seem only a reflection of variation in the number of mitochondria during regeneration and not of ATP-ase activity in the mitochondrion. Thus the fluctuations in the homogenate ATP-ase activity between the first and eighth day of regeneration may be attributed to the (S) fraction which contains the small granules (microsomes) and the soluble part of the cell and also to the variation in the number of mitochondria.

The ATP-ase activity of the liver cell and its distribution in the cellular components during stimulated growth process occurring after partial hepatectomy is essentially different from ATP-ase activity of the liver tumor during cancerous growth. The investigation of ATP-ase activity during azodye carcinogenesis is near completion and will be published at an early date.

Acknowledgments

We wish to extend our gratitude to Miss Denise Plante for her technical assistance and to Mr. Yvon Grandchamp, Statistician.

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A RAPID METHOD FOR THE DETERMINATION OF SODIUM IN SERUM[1]

By H. J. Suderman and G. E. Delory

Abstract

A colorimetric method for the determination of serum sodium is described in which sodium is precipitated from the deproteinized serum by uranyl zinc acetate under conditions chosen to minimize the time required. After washing, the precipitate is taken up in water and the amount of color developed on the addition of sodium salicylate is measured.

Introduction

Despite the development of the flame photometer, the need still exists for a rapid colorimetric method for the determination of serum sodium especially in smaller hospital laboratories. Of the many procedures published in recent years, most involve precipitation of sodium as the sodium zinc uranyl acetate salt as first suggested by Barber and Kolthoff (1). The washed precipitate is then redissolved and converted into a suitable derivative for colorimetric analysis.

In 1949, Stone and Goldzieher (7) published a method in which the time needed for the precipitation was reduced to 20 min.; a considerable improvement on earlier methods, for example that of King, Haslewood, Delory, and Beall (6) in which two hours were required. To obtain a derivative suitable for colorimetric analysis, Stone and Goldzieher used hydrogen peroxide in ammonium carbonate solution which gives a reddish brown color with uranyl ion, but which has a tendency to form bubbles along the walls of the colorimeter cuvettes. They suggested that, where large numbers of determinations were to be made, the bubble nuisance could be eliminated by adding the peroxide to a few tubes at a time. In our experience however this precaution proved to be inadequate as bubbles often formed immediately and the determination was thereby rendered useless. We did, however, confirm the superiority of their precipitation reagent.

It was therefore decided to attempt to modify this method by using some other derivative for colorimetry. One of the reactions most commonly used for this purpose depends upon the formation of a plum-red complex with potassium ferrocyanide in dilute acetic acid solution. However it is now appreciated that the color intensity of this complex increases with time and consequently errors may arise in routine use (4).

In a search for a better reagent we found that the uranyl ion when treated with salicylate gave a deep orange derivative which obeyed Beer's law, the quantities of sodium zinc uranyl acetate corresponding to the amounts of sodium (200–400 mgm. per 100 ml. of serum) likely to be encountered clinically.

[1] *Manuscript received March 10, 1952.*

Contribution from the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Man.

Experimental

To facilitate presentation the procedure as finally decided upon will be given first.

Reagents

Uranyl Zinc Acetate

Add 14 ml. of glacial acetic acid to 750 ml. of distilled water, bring to the boil, and add 77 gm. of uranyl acetate. To this mixture slowly add 231 gm. of zinc acetate with constant stirring. When solution is complete add 7 ml. of glacial acetic acid, cool to room temperature, dilute to one liter, and add 200 ml. of 95% ethanol. Usually, owing to sodium impurity in reagents, a precipitate forms upon standing overnight. If, however, no precipitate is seen, add a small amount of triple salt, prepared as below, to ensure saturation at all times. This solution must be filtered immediately before use. This reagent is similar to that of Stone and Goldzieher but to avoid the danger of re-solution of small amounts of sodium uranyl zinc acetate, the reagent is saturated with the triple salt.

Triple Salt

To 10 or 15 ml. of the above solution add enough of a concentrated sodium chloride solution to remove all but a trace of yellow color from the supernatant liquid. After 15 min., filter off the precipitate in a sintered glass crucible, wash five times with glacial acetic acid and five times with ether, then dry for about one hour in a desiccator.

Wash Reagent

Add 75 ml. of glacial acetic acid to 425 ml. of 95% ethanol and saturate with pure triple salt (see above) at room temperature. Store in a brown bottle; shake and filter before use.

Standard Sodium Chloride Solution (contains 350 mgm. of sodium per 100 ml.)

Dissolve 0.8896 gm. of sodium chloride in distilled water and make up to 100 ml.

Trichloroacetic Acid (10 gm. per 100 ml.)

Sodium Salicylate (10 gm. per 100 ml.)

Detailed Procedure

Deliver 0.5 ml. each of serum,* standard sodium chloride solution, and water into separate test tubes. To each add 10.0 ml. of 10% trichloroacetic acid and mix thoroughly. Filter the serum solution. From this point on the procedure is the same for all solutions. Transfer 1.0 ml. of the diluted protein-free solution into a centrifuge tube and add 3.0 ml. of uranyl zinc acetate reagent. Mix thoroughly by twirling the tube between thumb and forefinger.

** Proportionately smaller volumes of serum and trichloroacetic acid can be used when needed, for example, 0.1 ml. of serum and 2 ml. of trichloroacetic acid solution. The remainder of the procedure is unchanged.*

Allow to stand for 20 min., during which time the tube is twirled twice to stir up the precipitate. Centrifuge for seven minutes at 2000 r.p.m., then decant the supernatant and briefly drain the tube in an inverted position on a pad of filter paper. Add 3.0 ml. of alcoholic wash reagent, taking care to wash down the sides of the tube. Mix, centrifuge, decant, and drain thoroughly. Dissolve the precipitate in 14.0 ml. of distilled water, add 1.0 ml. of the sodium salicylate solution, and finally read the color in a photoelectric colorimeter, using a blue filter, or a spectrophotometer set to a wave-length of 460 mμ.

Calculation

$$\text{Serum sodium (mgm. per 100 ml.)} = \frac{D_T - D_B}{D_S - D_B} \times 350$$

where D_T, D_S, and D_B represent the optical densities of the test, standard, and blank respectively.

Stability of Colored Compound

The intensity of the colored solution produced by the addition of sodium salicylate to a solution of uranyl zinc sodium acetate was measured at intervals over a period of one hour and compared with the effect of time on the solution of uranyl ferrocyanide produced by the method of King *et al.* The results of a typical experiment are shown in Fig. 1.

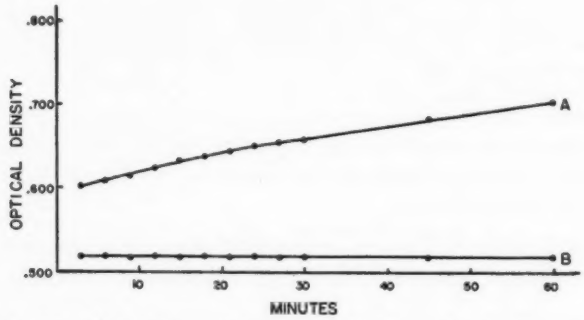

FIG. 1. The effect of time on the optical density of uranyl zinc sodium acetate solutions after treating with (*a*) potassium ferrocyanide (Curve *A*), and (*b*) sodium salicylate (Curve *B*).

Accuracy of the Proposed Method

The accuracy of the method was tested by (*a*) comparison with a standard gravimetric procedure, and (*b*) recovery experiments. Finally (*c*), values obtained by this method were compared with those obtained on the same samples of serum by a flame photometer method.

(*a*) *Comparison With the Gravimetric Method*

Samples of serum received in the Biochemical Laboratory of the Winnipeg General Hospital were analyzed by the proposed method and by the gravimetric procedure of Butler and Tuthill (2). The serum had been separated

from the cells without haemolysis (and as quickly as possible). Some of the samples were dialyzed against running water for two hours prior to analysis in order to obtain a low concentration such as might be encountered clinically, as for example in Addison's disease.

(*b*) *Recovery Experiments*

Varying amounts of sodium chloride were added to (i) undialyzed serum and (ii) serum which had been dialyzed to remove most of the sodium originally present. The sodium content of all these samples was determined by the proposed method.

(*c*) *Comparison With a Flame Photometer*

Samples of serum were divided into two parts, one sample was analyzed by the method described above and the other was analyzed by Dr. Marion Ferguson using a Barclay internal standard Flame Photometer.

Results

A comparison of values obtained on 10 samples of serum by the proposed method and by the gravimetric method are shown in Table I. With one exception all the colorimetric values are within 1.5% of the gravimetric values.

TABLE I

COMPARATIVE SODIUM ANALYSES ON HUMAN SERUM BY THE GRAVIMETRIC METHOD OF BUTLER AND TUTHILL (2) AND BY THE PROPOSED COLORIMETRIC METHOD

Concentration of sodium in mgm. per 100 ml. of serum		% Difference $\frac{A - B}{B} \times 100$
Colorimetric method (*A*)	Gravimetric method (*B*)	
250	254	−1.3
320	322	−0.6
329	325	+1.2
322	327	−1.5
331	329	+0.6
342	332	+3.0
334	334	0
334	335	−0.3
346	345	+0.3
354	349	+1.4
		Mean +1.0

The results for some typical recovery experiments are given in Table II. The differences between the amount of sodium found (in milligrams per 100 ml.) and the calculated amount (expressed as a percentage of the latter) varied between +1.1 and −1.8.

TABLE II

RECOVERY BY THE PROPOSED METHOD OF SODIUM ADDED TO SERUM

	Mgm. of sodium added per 100 ml. serum	Mgm. of sodium found per 100 ml. of serum (A)	Mgm. of sodium calculated (B)	Per cent difference $\frac{A - B}{B} \times 100$
Dialyzed serum	0	102	—	—
	200	297	302	−1.6
	250	348	352	−1.1
Dialyzed serum	0	25.7	—	—
	250	279	276	+1.1
	300	320	326	−1.8
Serum	0	345.5	—	—
	39.35	386	385	+0.3
	78.70	421	425	−0.9
	118.1	459	464	−1.1
	157.4	505	503	+0.4

Table III shows the results obtained in the comparison with the flame photometer method. While here, too, agreement is reasonably satisfactory, the spread was greater than in the comparison with the gravimetric method, the largest single difference (expressed as a percentage of the corresponding

TABLE III

COMPARATIVE SODIUM ANALYSES OF NORMAL HUMAN SERA BY A FLAME PHOTOMETRIC METHOD AND BY THE PROPOSED COLORIMETRIC METHOD

Concentration of sodium in mgm. per 100 ml. of serum		Difference $A - B$
Flame photometric method (A)	Colorimetric method (B)	
303	304	− 1
298	308	−10
314	309	+ 5
306	313	− 7
340	325	+15
323	325	− 2
344	325	+19
343	329	+14
340	330	+10
328	331	− 3
320	331	−11
352	337	+15
346	338	+ 8
337	339	− 2
	Mean difference	+ 3.6

flame photometer value) was 5.5%. It may be mentioned in this connection that Fox (5) also found differences up to 5.5% when comparing values obtained with his flame photometer with those obtained by the gravimetric method of Consolazio and Dill (3).

Discussion

The results which have been presented show that the proposed method is capable of an accuracy well within the requirements of clinical biochemistry. Furthermore, amounts of serum as small as 0.1 ml. may be used which is highly desirable when a complete study of electrolyte balance is desired and the volume of blood available is limited as, for example, with infants. Again the method is not only reasonably rapid but simple enough to be performed without special training on the part of the analyst. It has proved its value in the biochemistry laboratory of the Winnipeg General Hospital where it has been in routine use for 12 months.

Acknowledgments

We are greatly indebted to Dr. Marion Ferguson of the Department of Physiology and Medical Research for performing the serum sodium analyses with her flame photometer, to Dr. P. Green of the Deer Lodge Hospital, Winnipeg, who first suggested to us the use of sodium salicylate for the present purpose, and to Dr. F. D. White for his interest.

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THE EFFECTS OF SOME DIETARY DERIVED LIPIDS AND FAT SOLUBLE VITAMINS ON RAT SERUM TRIBUTYRINASE AND ALKALINE PHOSPHATASE[1]

By Jack D. Taylor, Neil B. Madsen, and Jules Tuba

Abstract

Synthetic diets were fed to adult rats for four weeks to determine the effects of dietary stearic acid, oleic acid, glycerol, Crisco, and vitamins, A, D, and E on the activity of serum alkaline phosphatase and serum tributyrinase. On a diet devoid of fats or fatty acids, the rats manifested abnormally low enzyme levels, which for serum alkaline phosphatase fell to values characteristic of starvation. Basal levels of the two enzymes, obtained with a fat free diet, were not altered by the ingestion of glycerol or vitamins A, D, and E. Dietary stearic acid, oleic acid, and Crisco, each significantly increased activity of phosphatase and tributyrinase and it would appear that both enzymes are concerned with intestinal absorption of fatty acids. The effect of oleic acid was most pronounced with both enzymes. The rats all gained weight during the tests so none of the variations in enzyme levels can be attributed to inanition. After the dietary test periods, all groups were starved for one week. Serum phosphatase values fell to the same constant low levels for all animals. Tributyrinase values rose towards levels which suggest that the enzyme is concerned with mobilization of depot fats during periods of fasting.

Introduction

The recent work of Madsen and Tuba (3) has shown that the abnormally lowered but constant levels of serum alkaline phosphatase that are characteristic of fasting rats may originate in various tissues, including the intestine. However, the increases above fasting levels of the serum enzyme in rats receiving a normal or a high fat diet, as well as in alloxan diabetic animals, appear to be derived chiefly from the intestine. It has also been established that there is a highly significant correlation between serum alkaline phosphatase activity and the daily consumption of food, particularly fat (1). Since these increases in the serum enzyme over the low, basal fasting values apparently arise in the intestine, the problem of the functions of intestinal phosphatase might be approached by determining which dietary components, particularly the constituents of the triglycerides, are responsible for the increased formation of the intestinal enzyme as reflected in the serum.

The earlier experiments of Weil and Russel (7) had shown that only the alcohol–ether soluble portion of Purina Dog Chow was effective in raising the plasma alkaline phosphatase values of adult rats above the lowered fasting levels. They tested the effect of a large number of substances upon fasting phosphatase levels, and found that the feeding of only a few unsaturated fatty acids or of cephalin was followed by the restoration of enzyme activity. Since it is now known that the increased serum alkaline phosphatase activity which results from the feeding of fats is derived from the intestine (3), it seems probable that the enzyme functions in the absorption of some fatty

[1] *Manuscript received March 18, 1952.*

Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council, Ottawa, Canada.

acids. We decided to reinvestigate the problem with larger groups of animals, and to feed them a basal fat-free diet for several weeks together with supplements of various derived lipids (glycerol, stearic acid, and oleic acid) and the fat soluble vitamins A, D, and E.

It has been shown by Tuba and Taylor (6) that the amount of hydrogenated vegetable fat consumed daily by adult male rats affected the levels of serum tributyrinase. Unpublished data obtained in this laboratory indicate that the nature of ingested triglyceride also influences the serum levels of this enzyme. It was decided that in conjunction with the phosphatase experiments the effects of the same derived lipids and fat-soluble vitamins on serum tributyrinase levels should be determined.

Experimental

Adult male albino rats weighing at least 250 gm. were housed in individual cages and given food and water ad libitum. The daily food consumption, body weight, and the levels of serum tributyrinase and alkaline phosphatase were determined for each animal after they had received Purina Fox Checkers for one week. After these initial values had been established, the animals were placed in groups of six on the diets indicated below, for periods of four weeks, and at the end of each week the various experimental data were again obtained, as well as at the end of the fifth week, during which interval of time the animals were starved.

Blood was obtained from the animals by bleeding them from the tail. Serum phosphatase was determined by the micromethod of Shinowara, Jones, and Reinhart (4), as modified by Gould and Schwachman (2). The unit of phosphatase activity is defined by Shinowara *et al.* as "equivalent to one milligram of phosphorus liberated as phosphate ion during one hour of incubation at 37° C., with a substrate containing sodium-β-glycerophosphate, hydrolysis not exceeding 10% of the substrate and optimum pH of the reaction mixture for the alkaline enzyme at 9.3 ± 0.15." Serum tributyrinase activity was determined by the titrimetric micromethod devised by Tuba and Hoare (5). The tributyrinase activity of rat serum is equivalent to the number of milliliters of 0.025 *N* sodium hydroxide required to neutralize the butyric acid liberated from tributyrin by the enzyme contained in 100 ml. serum in 30 min. at pH 8.05 and at 37° C.

Diet I was a a basal, fat-free diet consisting of: 20% vitamin-free casein (Smaco), 76% sucrose, 4% McCollum's salt mixture, together with adequate supplements of choline, thiamine, pyridoxine, pantothenic acid, nicotinic acid, and riboflavin. Cod liver oil, which we usually add as a supplement for synthetic diets, was omitted from this basal diet, and the following levels of fat-soluble vitamins were added: 1.3 mgm. vitamin A acetate, 0.15 mgm. calciferol, and 133 mgm. alpha-tocopherol, per kilogram of diet. Diet II consisted of the basal diet, with the crystalline vitamins A, D, and E omitted,

in order to estimate the effect, if any, of these three substances on the serum enzymes. The remainder of the diets were devised to test the effect on the two enzymes of glycerol or fatty acid equivalent to the concentrations which would be present in a diet containing 10% triglyceride. In Diet III an equal amount of sucrose was displaced by 1.04% glycerol, which is equivalent to the amount of glycerol which would be present in a synthetic diet containing 10% tristearin. In Diets IV and V, equal amounts of sucrose were replaced by 9.57% stearic acid and 9.57% oleic acid respectively. These concentrations of the saturated and the unsaturated fatty acid are equivalent to the amounts which would be present in synthetic diets containing either 10% tristearin or 10% triolein. Diet VI, which served as a control, consisted of the basal diet in which 10% of a hydrogenated vegetable fat, Crisco, replaced an equal weight of sucrose.

Results

The effects of the six diets on the levels of rat serum alkaline phosphatase and serum tributyrinase are indicated in Table I. The enzyme values are

TABLE I

THE EFFECTS OF DIETS I – VI ON SERUM ALKALINE PHOSPHATASE (UNITS/100 ML.) (P), SERUM TRIBUTYRINASE (UNITS/100 ML.) (T), DAILY FOOD CONSUMPTION (GM.) (C), AND ON BODY WEIGHTS (GM.) (ΔW) OF ADULT MALE RATS. (MEANS OF SIX ANIMALS)

Diet	Zero values		Means of four weekly values during test				After one week of postdietary fasting	
	P	T	P	T	C	ΔW	P	T
I	74 ± 5*	650 ± 40	28 ± 1	290 ± 10	13.8 ± 0.5	+ 52 ± 7	27 ± 2	400 ± 30
II	88 ± 8	630 ± 30	28 ± 1	290 ± 10	14.0 ± 0.5	+ 38 ± 7	33 ± 3	420 ± 20
III	90 ± 3	650 ± 20	27 ± 1	280 ± 10	15.5 ± 0.4	+ 37 ± 4	29 ± 3	390 ± 20
IV	80 ± 6	680 ± 20	43 ± 2	370 ± 20	12.8 ± 0.4	+ 31 ± 4	33 ± 4	420 ± 20
V	77 ± 14	650 ± 20	80 ± 2	440 ± 10	13.6 ± 0.4	+ 50 ± 6	31 ± 2	490 ± 30
VI	90 ± 5	660 ± 20	75 ± 2	350 ± 10	13.5 ± 0.4	+ 46 ± 3	33 ± 1	390 ± 20

* *Standard error of the mean.*

given at the beginning of the feeding experiment, and then the means of the four weekly values obtained during the test period are presented, as well as levels at the conclusion of the fasting period of one week. The average daily food consumption per rat over the four week feeding period is given for each diet. The changes in body weight which occurred as a result of feeding each diet for four weeks are also included, and it is interesting to note that in every case the animals gained weight.

Weekly fluctuations in the activities of serum alkaline phosphatase and serum tributyrinase are shown in Figs. 1 and 2 respectively.

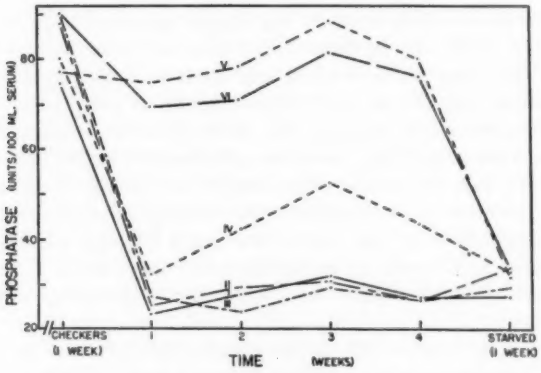

FIG. 1. The mean serum alkaline phosphatase levels of groups of rats as a result of (*a*) a pre-experimental diet of powdered Purina Fox Checkers for one week, (*b*) test diets I-VI for four weeks, and (*c*) postdietary starvation for one week.

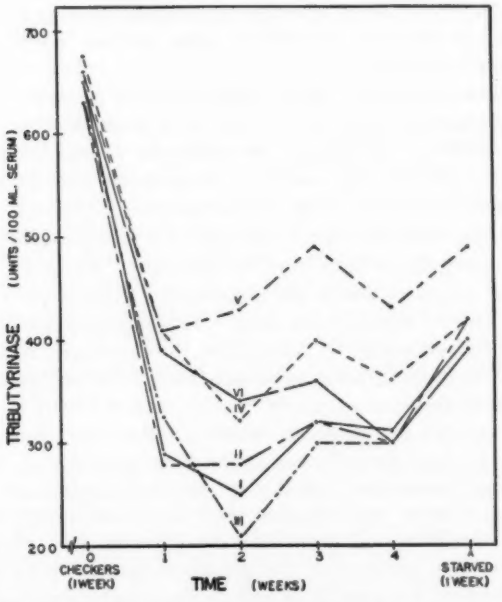

FIG. 2. The mean serum tributyrinase levels of groups of rats as a result of (*a*) a pre-experimental diet of powdered Purina Fox Checkers for one week, (*b*) test diets I-VI for four weeks, and (*c*) postdietary starvation for one week.

Discussion

There are no significant differences in food consumption by the various groups which could account for variations in enzyme levels. There is no statistically significant difference in the weight gains of the six groups of animals after four weeks on the diets, despite the fact that the first three diets are fat free. This may be taken as an indication that food consumption was adequate and that the animals were in good health in every case. This is of particular importance with regard to rat serum alkaline phosphatase because we have consistently found that inanition and loss of weight result in abnormally low activity of the enzyme. The weight gain on every diet, therefore, indicates that changes in this enzyme are directly attributable to dietary factors. The animals must also have had ample reserves of the three fat-soluble vitamins, A, D, and E, and of essential fatty acids to carry them through the test periods when they were receiving diets lacking these nutritional factors.

An analysis of variance of the serum alkaline phosphatase levels for the four weeks on the test diets indicates that there is a highly significant variation with some diets. There is, however, no significant difference between the values for Diets I, II, and III. Furthermore, since starvation for one week does not alter the serum phosphatase levels of the rats on these diets, it may be said that the three fat-soluble vitamins, glycerol, and the nonlipid portion of the diet do not affect the fasting levels of the enzyme in serum. This result agrees with those of Weil and Russel (7), who did not, however, test the effect of fat-soluble vitamins.

One week of postdietary starvation reduced serum phosphatase levels of the six groups of animals to practically the same basal level. There is, in fact, no significant difference between the values for any of the groups, and it may therefore be said that the previous dietary history of the animals has no effect on the starvation level of the serum enzyme. This is in conformity with the observations of Madsen and Tuba (3) that serum phosphatase activity above this level is largely derived from the intestine. Diets IV, V, and VI produced enzyme activities which are significantly greater than starvation levels or levels on the first three fat-free diets. This emphasizes the importance of phosphatase in fat absorption by the intestine. The stearic acid of Diet IV increased the activity of serum alkaline phosphatase above the basal starvation level, although not to the same extent as the oleic acid of Diet V. The stearic acid effect, however, did not show up at once, and in fact the serum phosphatase after one week on Diet IV was at the starvation level. We have no explanation for this observation, but it may very well explain why Weil and Russel (7) did not observe any effect of this fatty acid in their short term experiments. It would appear from our experiments that alkaline phosphatase is in some way involved in the intestinal absorption of stearic and possibly other saturated fatty acids, as well as the unsaturated fatty acids.

Both oleic acid and Crisco produced serum phosphatase values comparable with the normal values on Checkers (Table I and Fig. 1). The phosphatase

activity produced by dietary oleic acid is slightly but significantly greater than the activity on the Crisco diet. These results agree with those of Weil and Russel (7).

The serum tributyrinase values of the animals receiving the first three diets are the lowest of the six groups, and they do not differ significantly from each other. A fat-free diet therefore results in a profound decrease of enzyme activity from the levels found in animals receiving the stock laboratory ration, and this change is not modified by the absence of the fat soluble vitamins, A, D, E, from Diet II or by the addition of glycerol to Diet III.

Stearic acid (Diet IV) and Crisco (Diet VI) produce serum tributyrinase activity which is in each case significantly greater than what may be considered a basal value for the three fat-free diets. The enzyme levels for Diets IV and VI do not differ significantly from each other. It is of importance to note that values for these two diets are not as great as those obtained with all six groups during the initial period on Purina Fox Checkers. Since the Checkers contain approximately 5% total "fat", there must be present lipids or other factors which affect the serum tributyrinase activity, and therefore levels of the enzyme in serum of animals receiving this diet cannot be accounted for on the basis of ingestion of triglycerides or saturated fatty acids alone.

The use of oleic acid as a supplement with the basal diet produced the greatest elevation of tributyrinase as well as phosphatase activity. The values with Diet V are significantly different from all the other groups, and although fatty acid equivalent to 10% triglyceride is present in the Diet, enzyme activity is again not as high as with Checkers.

The effect of postdietary starvation on serum tributyrinase is not identical with all groups as in the case of serum phosphatase. However, in each case starvation has produced a definite increase in serum tributyrinase hydrolyzing activity, although these changes are significant only in Groups I, II, III, and IV. The effect of starvation on the enzyme has been previously noted in other experiments reported from this laboratory (5, 6). It is very likely associated with the mobilization of depot fat during the period of fasting. The variations between fasting values of the six groups used in this experiment and those reported in the two previous papers (5, 6), are probably due to differences in fatty acid content of depot fats formed on the various dietary regimens.

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ACUTE EXPERIMENTAL HEART FAILURE PRODUCED BY INTRAVENOUS GUM ACACIA INFUSION[1]

By C. W. Gowdey and J. S. Loynes[2]

Abstract

This paper describes some of the cardiovascular changes produced in anaesthetized dogs by slow, continuous, intravenous infusion of gum acacia solution. As the blood volume increased and the haematocrit fell, the cardiac output and heart size increased. The right and left auricular pressures rose slowly at first, then more rapidly as the venous system became more distended. A point was reached at which the heart, unable to cope with the ever-increasing load, began to fail. The intracardiac pressures and cardiac size increased rapidly; cardiac arrhythmia developed, and death followed within half an hour. Observations comparable to those in clinical congestive heart failure included the following: a greatly enlarged heart, high intracardiac pressures, hepatic engorgement, occasionally peripheral oedema, maintenance of arterial blood pressure until frank decompensation occurred, and decreased urinary flow coinciding with the reduced cardiac output.

Introduction

The physiology of acute hypervolaemia in dogs has been studied preparatory to an investigation into the mechanism of action of digitalis. Because an increased blood volume is a common clinical finding in decompensated patients (13), it was hoped that an investigation into the effects of slow, continuous, intravenous infusions of plasma substitutes or blood might add to the knowledge of the congestive state. Furthermore, it was hoped that such a method might prove more suitable than the heart–lung preparation for studying the effects of digitalis on animals.

In 1922, Wiggers and Katz (19) found that rapid saline infusions in dogs caused an increase of right atrial pressure which, above a critical level, was associated with cardiac decompensation. Other investigators (18, 21) produced some of the phenomena of congestive failure—increased heart size and rate, gallop rhythm, congestion of liver and lungs, and sometimes pulmonary oedema—by massive infusions of saline or glucose.

In preliminary reports from this laboratory (4*a*, *b*) it was shown that a continuous infusion of gum acacia solution in the open-chested dog increased the filling pressures, size, and work of the heart. Eventually, reduction of the cardiac reserve and acute heart failure occurred. Later, Huckabee, Casten, and Harrison (6) reported heart failure in some of the dogs made hypervolaemic with infusions of albumen, heparinized horse blood, or dog's blood.

The present paper deals with the cardiovascular adjustments to hypervolaemia and attempts to define suitable criteria for acute, experimental heart failure; the effects of digitalis in this type of failure will be reported in a later paper.

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Procedure

Adults dogs weighing between 10 and 22 kgm. were anaesthetized by the intravenous injection of pentobarbital sodium. A suitable plane of anaesthesia was maintained by intramuscular pentobarbital as required. The arterial blood pressure was recorded by a mercury manometer from a cannula in either the brachial or femoral artery. The brachial vein was cannulated and connected to an intravenous set for the infusion. A cannula was tied in the trachea, the chest opened, usually along the midline, and artificial respiration with air begun. To record right auricular pressure (RAP), a polyethylene catheter connected to a saline manometer was pushed down an external jugular vein into the right auricle. A similar catheter was introduced into the left auricle through one of the pulmonary veins to measure the left auricular pressure (LAP).

A cruciform incision was made in the pericardium, and ligatures tied around the four pericardial flaps thus produced. A Henderson cardiometer was inserted through the incision, fitted over the ventricles, and made airtight by tying the pericardial flaps around it. The cardiometer was then attached to a float recorder of low-frequency response through a glass capillary resistance to dampen the excursions further. A Marey tambour of high-frequency response was placed in parallel with the float recorder. This recording system allowed the slow-moving float recorder to change with changes in mean cardiac size, while maintaining the tambour at a constant pressure to measure the stroke output.

Samples for haematocrits were drawn from the right auricular catheter, and measured in Wintrobe tubes. Heart rates were taken from the kymographic tracing. The bladder was exposed and a glass cannula tied in place; then the abdominal incision was carefully closed around the cannula. The urine thus collected was measured in a graduated cylinder. The pre-infusion arterial blood pressure, RAP, LAP, mean cardiac volume, heart rate, stroke output, haematocrit, and urine flow were recorded. Not all measurements were made, however, in all experiments. Then a continuous infusion of isosmotic gum acacia solution was begun. The concentration of the gum acacia was 6%, dissolved in 0.9% saline; the solution was buffered to pH 7.0–7.2 with sodium bicarbonate, and was then filtered. Readings of the rate of infusion, and of the above variables were usually taken at 10-min. intervals except near the point of heart failure when more frequent observations were made.

Results

Twenty-one experiments with gum acacia infusions have been performed, the average rate of infusion being 0.6 ± 0.1 ml. per kgm. per minute. Fig. 1 is the tracing obtained in a typical experiment using gum acacia to increase the venous return. The infusion began at the arrow, and was continuous throughout the experiment at an average rate of 0.6 ml. per kgm. per minute. Soon after the infusion began, the arterial blood pressure climbed towards the level recorded before the chest was opened, and the volume of the heart began to

increase. This increase in cardiac size was accompanied by an increased stroke output, which in Panel 6, after three hours of infusion, was 3.4 times the control, pre-infusion level. With the ever-increasing venous return, the filling pressures in both the right and left auricles began to rise. It can be seen that the left auricular pressure began to rise before the right, and rose more sharply. A point was reached at which the heart was incapable of dealing with the excess load and it began to fail. The arterial blood pressure and stroke output began to fall, while the cardiac size and left and right auricular pressures rose very rapidly. Within seven minutes the heart became arrhythmic, the stroke output fell to zero, and the dog died. This animal was infused with 1 liter of acacia solution which was equivalent to its total calculated blood volume, if a blood volume of 90 ml. per kgm. body weight is assumed.

Tables I and II show abbreviated protocols for 21 experiments with gum acacia infusions. Control data are listed opposite time zero; the other time figures are elapsed times from the beginning of infusion. The percentage increase in stroke output over the control level, the changes in auricular pressures, cardiac size, arterial blood pressure, urine flow, haematocrit, and the mean rate of infusion are shown. Table I includes 11 experiments in which gum acacia solution was infused until the death of the animal, and one (Dog 5) in which the infusion was stopped after 190 min. In Table II the results of nine experiments are summarized in which, before the stroke output had begun to decrease, a cardiotonic drug was given. No data obtained after such an injection are included in the table. The effects of cardiotonic drugs in these and other experiments will be reported in detail at a later date. Table II is included because, it too, illustrates some of the cardiovascular adjustments to an increased blood volume.

It can readily be seen that the gum acacia infusion usually caused a great increase in the stroke output. It is not understood why, in Experiment 18, the auricular pressures, cardiac output, and cardiac size all decreased, even though the haematocrit decreased as usual. In Dogs 5 and 12 the stroke output, arterial blood pressure, and auricular pressures are seen to decrease when the infusion was stopped. In Dog 12 the haematocrit was also found to rise and the urine flow to decrease, after the infusion had been interrupted. When the infusion no longer produced an increase in stroke output, but rather a decrease, this decrease was associated with a marked rise in auricular pressures and size of the heart. The failure progressed with surprising rapidity until death occurred. In this paper the term "failure" is used to denote that point at which the stroke output began to fall, and kept falling, in spite of rising auricular pressures and increasing heart size.

The variations in heart rate in all of the experiments were less than 10% of the pre-infusion rate until frank failure developed, during which arrhythmias often occurred. For this reason the changes in stroke output and minute output were essentially similar until the heart failed.

These experiments show that with the beginning of infusion the arterial blood pressure started to rise rapidly towards the level recorded before the

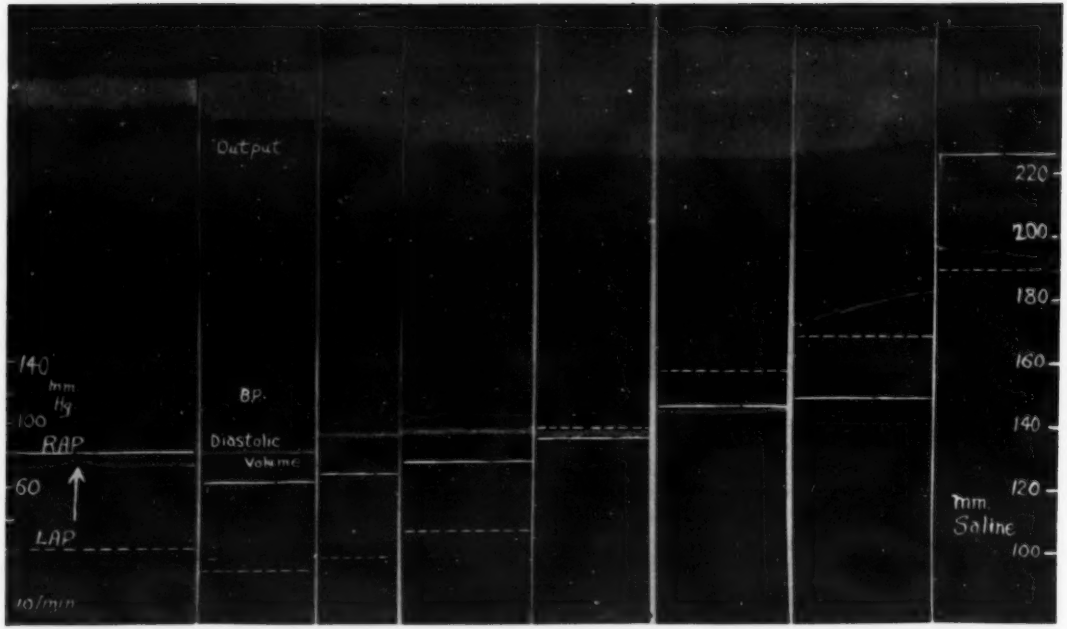

FIG. 1. Cardiovascular effects of intravenous acacia infusion. Dog, 11 kgm.: Infusion began at arrow and was continuous throughout experiment at rate of 0.3 ml. per kgm. per min. From above downwards: stroke output, arterial blood pressure, right auricular pressure (RAP), diastolic volume, left auricular pressure (LAP), baseline for arterial pressure—the zero for atrial pressures was table top level. Time = 6 sec. Total acacia infused = 1000 ml.

TABLE I

SUMMARY OF EFFECTS OF GUM ACACIA INFUSIONS

Expt. and rate of infusion[1]	Time (minutes of infusion)	% Inc. in stroke output over control	Change in RAP (mm. saline)	Change in LAP (mm.)	Change in cardiac size (cc.)	Arterial B.P. (mm. Hg)	Urine flow (ml./min.)	Haematocrit
No. 1	0					100		
13 kgm. f.	120	38	60		20	126		
0.9 ml./kgm./min.	150	88	70		50	118		
	180	150	220		120	78		
	196	−50	300		135	10		
No. 2	0					72		
17 kgm. m.	60	125	20		15	80		
1.1 ml./kgm./min.	90	150	25		20	54		
	115	−50	155		95	5		
No. 3	0					76		
10 kgm. f.	30	100	40		4	88		
0.9 ml./kgm./min.	60	600	220		101	84		
	118	400	280			10		
No. 4	0					64		
20 kgm. f.	30	90	250			100		
1.4 ml./kgm./min.	40	87	335			82		
	45	85	315			30		
No. 5	0					76		
9 kgm. f.	60	20	10			104		
1.1 ml./kgm./min	90	22	5			100		
	180	74	40	Acacia off from 190 min.		74		
	220	−2	2			40		
No. 7	0					140	0.1	
13 kgm. f.	75	155	9		1	160	0.6	
0.5 ml./kgm./min.	135	285	64		3	122	0.9	
	165	270	33		5	102	0.2	
	195	−70	130		106	14	0	

[1] *Weight and sex of dogs also given.*

TABLE I—*Concluded*

SUMMARY OF EFFECTS OF GUM ACACIA INFUSIONS—*Concluded*

Expt. and rate of infusion[1]	Time (minutes of infusion)	% Inc. in stroke output over control	Change in RAP (mm. saline)	Change in LAP (mm.)	Change in cardiac size (cc.)	Arterial B.P. (mm. Hg)	Urine flow (ml./min.)	Haematocrit
No. 8	0					100	0.1	
14 kgm. m.	90	80	5		20	120	0.3	
0.4 ml./kgm./min.	245	150	31			106	0.5	
	335	320	37		70	107	2.3	
	385	280	118		155	30	1.6	
No. 9	0					65		
11 kgm. f.	60	93	5	12	22	124		
0.5 ml./kgm./min.	120	180	7	27	27	106		
	180	350	28	88	45	56		
	187	115	105	109	95	10		
No. 17	0					92	0.2	44
18 kgm m.	60	100	23	−11		104	0.3	
0.6 ml./kgm./min.	120	186	31	39		102	1.5	20
	150	176	34	48		90	1.2	
	174	93	167	36		32	0.3	
No. 18	0					60	0.02	46
18 kgm. m.	60	−4	−18	−10	−10	56	0.3	
0.5 ml./kgm./min.	80	−31	−17	−10	−15	40	0	33
No 19	0					144		50
15 kgm. m.	120	106	−37	−4		140		
0.5 ml./kgm./min.	180	233	−28	2		140		
	240	380	67	94		140		22
	280	368	85	110		116		
No. 20	0					74		50
12 kgm m.	60	86	1	61	11	93		
1.1 ml./kgm./min.	110	95	1	83		72		14
	130	90	1	98	13	67		

[1] *Weight and sex of dogs also given.*

TABLE II

EARLY STAGES OF FURTHER INFUSION EXPERIMENTS

Expt. and rate of infusion[1]	Time (minutes)	% Increase in stroke output over control	Change in RAP (mm. saline)	Change in LAP (mm.)	Change in cardiac size (cc.)	Arterial B.P. (mm. Hg)	Urine flow (ml/.min.)	Haematocrit
No. 6	0					94		
18 kgm. m.	30	48	42			106		
0.3 ml./kgm./min.	65	107	52			90		
	120	124	92			100		
No. 10	0					78	0.01	53
13 kgm. m.	60	18	0	29	7	100	0.1	
0.3 ml./kgm./min.	90	23	5	34	17	102	0.1	
	130	59	10	42	25	104	0.2	34
No. 11	0					106	0	54
15 kgm. m.	70	114	0	23	10	105	0.01	48
0.3 ml./kgm./min.	100	128	−6	26	15	122	0	
	170	171	−7	43	16	102	0	32
No. 12	0					129	0.2	54
17 kgm. m.	60	64	−22	23		130	0.2	45
0.2 ml./kgm./min.	180	86	13	24		142	1.0	35
	240	122	20	35		138	1.4	
	300	163	24	44		133	1.4	
Inf. off 332	330	186	29	51		130	1.7	26
	395	154	19	38		120	0.6	
Inf. on 445	440	127	17	38		117	0.1	33
	475	154	20	42		118	0.2	32
No. 13	0					112	0.03	54
20 kgm. m.	80	50	3	−19		113	0.1	31
0.5 ml./kgm./min.	170	25	12	−9		86	0.1	
	235	18	34	44		62	0	18
No. 14	0					56		60
18 kgm. m.	70	6	14	15	48	93		50
0.4 ml./kgm./min.	145	15	26	29		68		30
	210	22	44	52		50		23
No. 15	0					130	0.1	58
17 kgm. m.	75	147	36	22		120	0.4	31
0.4 ml./kgm./min.	105	152	35	25		110	0.7	29
	135	152	30	18		103	0.4	29
No. 16	0					116	0.01	54
21 kgm. m.	90	150	19	20	5	82	0.4	32
0.6 ml./kgm./min.	120	182	32	36	23	94	0.5	
No. 21	0					70		51
17 kgm. m.	30	62	17	57	12	106		
1.0 ml./kgm./min.	50	120	20	73	24	106		40

[1] *Weight and sex of dogs also given.*

chest was opened. During the later stages of the experiments, it was found that the arterial pressure decreased before the cardiac output. The urine flow, which was usually very small during the control period because of low blood pressure and blood loss, increased rapidly with the infusion. When the blood pressure began to fall, and failure became imminent, the urine flow was reduced markedly.

The RAP and LAP rose very slowly in the early stages of infusion even though the stroke output increased rapidly during this time. In fact, in 17 out of 21 experiments (81%), the RAP was less than 70 mm. of saline above the pre-infusion level at the time of failure. This occurred in spite of the fact that the output rose an average of 189% ± 34.5 (S.E. of mean of 21 experiments) above the pre-infusion level. In the later stages of the experiments the auricular pressures rose rapidly, and at the time of death, the mean level of RAP was 149 mm. saline above the pre-infusion level. In 10 out of 13 experiments (77%) the LAP rose sooner, and initially, more sharply than the RAP.

The stroke output appeared roughly to vary inversely as the haematocrit: as the haematocrit decreased, the stroke output increased up to the point of failure. Too few points were obtained in each experiment to establish this relationship definitely, but by taking the points (up to the time of failure or of cardiotonic administration) from all of the experiments together, an over-all coefficient of correlation between the stroke output and haematocrit of −0.7 was found. Haematocrits between 35% and 25% were sometimes associated with cardiac failure; failure usually occurred with haematocrits reaching 25% or less.

The mean cardiac size increased as the cardiac output rose. Just before heart failure occurred, the mean cardiac volume began to rise more quickly, and at the time of cardiac arrest reached up to 180 cc. above the pre-infusion size. It was found that, although variable, increases in heart size of more than 70 cc. over the pre-infusion level were usually associated with a decompensated heart. In individual experiments there was good correlation between cardiac output and cardiac size, but because of variability from one experiment to another the coefficient of correlation for all experiments taken together was 0.6.

On gross examination post mortem, a greatly dilated heart and distended systemic veins were regularly found. Occasionally there were a few small subpericardial haemorrhages; the coronaries were invariably patent. Many of the lungs showed moderate congestion, but gross pulmonary oedema was not evident. There was often a clear, straw-colored pleural effusion. The liver was engorged, the spleen was contracted, and occasionally there were small subserosal haemorrhages in the bowel. There was often 100–200 ml. of clear ascitic fluid. Gross examination showed nothing abnormal in the kidneys. Marked peripheral oedema was occasionally present.

Discussion

It can be seen from the tables that the mean pre-infusion arterial blood pressure in these experiments was 93 mm. Hg ± 27.6 (S.D.); in 12 of these dogs the control haematocrit was 52.4% ± 4.4 (S.D.). These figures are indicative of the blood loss and trauma which usually accompanied the open-chest technique. The hypotension was not usually severe enough, however, to stop urine flow. The blood pressure invariably rose soon after the infusion began, and remained within normal limits until just before decompensation occurred. This maintenance of blood pressure even during the early stages of heart failure is in accord with clinical findings.

The heart rate was always high under pentobarbital anaesthesia (140–180 per minute) and in these experiments varied so little that the stroke-output tracing gave a continuous measure of the cardiac minute output. In 'controlled circulation' experiments in dogs, Wiggers and Katz (19) increased the venous return by saline or blood infusions into an external jugular vein. In their experiments the heart rate was kept constant (below 80 per minute) by artificial stimuli, and the aortic resistance by an adjustable aortic clamp. They found that with a progressive increase in venous return, the venous and atrial pressures rose, the diastolic size of the heart increased, and the systolic discharge became greater. The diastolic aortic pressure was kept constant, but the systolic and pulse pressures increased. These changes progressed until a critical atrial pressure was reached. Then a circulatory crisis occurred; the diastolic ventricular size increased rapidly while the systolic stroke became progressively smaller. The minute output and systolic aortic pressure decreased rapidly. The heart had passed beyond its reserve power and began to decompensate.

In the experiments reported here, although the output rose very soon after the infusion began, the increased output was associated with small increases in RAP until near the end of the experiment. Indeed when the RAP began to rise rapidly, it was a sign of impending cardiac decompensation, failure and death usually occurring within 30 min. The preparation described here, which includes the peripheral circulation, differs in other important respects from the Knowlton–Starling heart–lung preparation (8). In the heart–lung preparation the venous blood is returned to the heart from the venous reservoir by relatively nondistensible rubber tubing rather than by a venous system whose capacity as well as its pressure is capable of wide variations. The cardiac output is equal to, and determined by, the venous inflow, and the output can vary widely according to the inflow. The arterial blood pressure in the heart–lung preparation is fixed by an artificial peripheral resistance, whereas in our experiments the blood pressure is under physiological control. Another difference is that in Starling's preparation the heart rate was dependent only on temperature and was not modified by cardiac reflexes. It was been shown (3, 7) that the maximum cardiac output in the heart–lung preparation is considerably less than that in the resting unanaesthetized dog. For these reasons it is felt that, although no open-chested dog can be considered normal,

at least these experiments are much more physiological than heart–lung preparations. It is true that after the infusion had continued for some time the RAP did begin to rise with the rising output, but early in the experiment large output increases were accompanied by very small changes in RAP. It would thus appear that the increasing output was made possible by an increased venous return, which early in the experiment occurred with little change in venous pressure, but which, as the venous bed became filled, caused an ever-increasing rise in RAP. Just how important these small increases in filling pressure are in determining the cardiac output during the phase of venous filling, is difficult to assess, but certainly the greatest changes in output occurred while the RAP rose only a few centimeters.

Stead and Warren (17), using the cardiac–catheterization technique in humans, concluded that in the presence of an adequate volume of blood, the cardiac output is varied by changes in ventricular relaxation and contraction which are independent of fairly wide variations in atrial pressure. Once the blood volume has been decreased sufficiently to decrease the output, then a small change in atrial pressure causes a great change in output. The atrial pressure changes may be scarcely measurable. Under such circumstances the changes observed parallel those reported for the heart–lung preparation. Huckabee *et al.* (6) found that a linear relationship between inflow load and cardiac output did not exist in the intact dog. If other factors remained constant, a minimal rise from the normally low level of venous pressure tended to be reflected in large increments in cardiac output. At high levels of venous pressure further increments had little effect.

In 10 out of 13 experiments the LAP rose sooner and more sharply than the RAP, or in other words the rise in LAP more closely paralleled the cardiac output than did the RAP. The differences observed between the left and right auricles in both onset and rate of pressure rise during the continuous infusion may be explained partly by recent experiments of Little (10). He studied the relation between atrial pressures and increments in atrial–caval volumes in isolated dog hearts with the A–V valves sutured. The right atrial system required twice the volume for initial filling as the left, and, even when filled, the right atrium was much more distensible than the left.

The cardiac volume increased as the cardiac output rose, and, as one would expect, in the early stages of infusion there appeared to be a linear relationship between them for individual experiments. Meek and Eyster (12) found that the diastolic size of the heart (measured with an X-ray technique) showed no increase, or only a very transient increase, after infusions of saline, acacia, or blood were given to dogs to the extent of 30–100% of their calculated blood volumes. In two dogs they observed, during infusion, a steady increase in diastolic size and cardiac output as the venous pressure increased from 3 to 130 mm. of water. Usually little or no increase in diastolic size was seen after the venous pressure reached 100 mm. Meek and Eyster believed however, that the different blood reservoirs of the body could accommodate large

increases in blood volume, and that the venous return and cardiac discharge were not, or only momentarily, increased.

From the experiments reported here it was found that there was an inverse correlation between the haematocrit and cardiac output up to the point of failure, and that the relation between atrial pressures and cardiac output was probably nonlinear. This suggests that the cardiac output may be determined less by the filling pressures themselves than by such factors as increased plasma volume and resulting dilution anaemia.

Many attempts have been made to produce congestive heart failure in experimental animals. Most of these involved direct physical or chemical damage to the myocardium: injection of toxic substances (chloral, diphtheria toxin, ethanol, etc.) (2), burning of the ventricle (16), ligation of the coronaries (14) or plugging them with starch grains (15), and even production of valvular lesions (9).

In the present method the heart is not damaged by direct physical or chemical means, and, although such factors as certain electrolyte changes may play a part, it seems probable that the hypervolaemic dilution anaemia plays a major role in causing the cardiac output to increase. When the haematocrit reaches such low levels that sufficient oxygen can no longer be delivered to the myocardium, then heart failure must result. This type of failure is somewhat comparable to the "high-output failure" described by McMichael (11) in certain anaemic patients. From the results presented here, it is not possible to draw unequivocal conclusions regarding the exact cause of the observed changes in cardiac output, but from preliminary experiments with whole-blood infusions (5) it seems probable than an increased volume of the circulation per se does not lead to high-output failure. The diminished oxygen capacity of the diluted blood may be very important in these experiments since it has been found that one of the important compensations to a decrease of the oxygen capacity of the blood during experiments with progressive hypoxia was an increased cardiac output (20).

The heart failure described above is similar in several respects to the clinical symptomatology designated as "congestive heart failure": there is an elevated right atrial pressure and a greatly dilated heart; the systemic veins, liver, and lungs are engorged. There may be peripheral oedema, and the arterial blood pressure is maintained within nearly normal limits until just before death.

Acknowledgment

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CARDIOTONIC ACTIVITY OF CERTAIN STEROIDS AND BILE SALTS[1]

By J. S. Loynes[2] and C. W. Gowdey

Abstract

The effects of 23 plant and animal steroids, steroid hormones, and bile acids, and nine of their salts or soluble conjugates, have been investigated in isolated frog hearts. All but five of the compounds produced significant augmentation of frog hearts made hypodynamic by prolonged perfusion. The augmentation was not usually accompanied by changes in heart rate, and no steroid caused systolic arrest. Eight water-soluble steroid salts were perfused through isolated rabbit hearts when they had become hypodynamic by prolonged perfusion. In each case the coronary flow increased significantly whether the heart rate and force of contraction increased or not. Thus, when cardiotonic activity was observed, it appeared to be a direct effect and not secondary to the increased coronary flow. It would seem that the lactone ring of the cardiac glycoside molecule is responsible for the development of systolic arrest, and that the cardiotonic action is, at least partly, a function of the cyclopentenophenanthrene nucleus.

Preliminary reports (10, 26) from this laboratory have shown that a number of steroids are capable of increasing the force of contraction of isolated perfused frog and mammalian hearts. This investigation was prompted by an observation by Waud (27) that phytosterols from the Osage orange caused augmentation of the perfused frog heart. Because the plant steroids are related chemically to cholesterol, this compound was tried, and it appeared to have a positive inotropic effect; no systolic arrest was observed. The cyclopentenophenanthrene nucleus of cholesterol is common to the plant steroids, the cardiac glycosides, the adrenal cortical and sex hormones, bile salts, and vitamin D compounds. It was decided, therefore, to study the cardiac action of a group of these steroids, and determine whether part of the activity of the cardiac glycosides might be related to their cyclopentenophenanthrene nucleus. The lactone part of the cardiac glycoside molecule has already (3, 8, 13) been shown to produce both an increased force of contraction and systolic arrest.

The literature revealed that relatively little is known of the cardiac action of the steroids. Several investigators have shown that cholesterol increases the force of contraction of the frog heart (4, 5, 7, 18). Horral and Carlson (12) found in man, however, that intravenous cholesterol caused no symptoms.

Various workers (2, 16, 24) have reported bradycardia in isolated frog and mammalian hearts, and in intact animals and man after certain bile salts. Decholin (dehydrochloric acid) was found to reduce the blood pressure of humans after several doses (1). Wakim and Essex (25) found in dogs that bile salts and acids slowed the heart rate, reduced the force of contraction, produced irregular rhythms, and finally failure. Rubin (17) reported that certain

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steroids of the estrogen and pregnane series produced augmentation of isolated frog hearts made hypodynamic by reducing the calcium concentration of the perfusion fluid.

Several papers (6, 11, 14, 15, 19, 22) have appeared recently, concerning the effects of androgens on angina of effort. Summers (23) found, however, that testosterone caused no significant changes in heart size, in the electrocardiogram, or in systolic blood pressure, and produced only transient improvement in some cases of angina.

Methods

The cardiotonic activity of the steroids was studied on the isolated frog heart and the isolated rabbit heart. The excised frog heart was perfused through the vena cava with Ringer's solution buffered to pH 7.0–7.2. The amplitude of cardiac contraction was recorded on a lightly-smoked drum by means of a pin, through the apex, connected to a light isotonic lever. The heart was perfused until the maximum initial amplitude had decreased by 30–50%; the usual time for this hypodynamic state to be reached was 60 to 75 min. The steroid was then perfused until after a maximal effect had been obtained, (average time 10 min.). The aqueous-soluble steroid salts were added to the perfusion fluid to final concentrations varying from 1 to 200 μgm. per ml. The relatively insoluble compounds were usually dissolved in 1 ml. of warm absolute alcohol, and added to the perfusion fluid to a final concentration of 10 μgm. per ml. A fine white suspension with a pH of 7.2 resulted. In some experiments the steroids were dissolved first in 1 ml. propylene glycol and then added; in others, microcrystals were added with no solvent, to control the effects of the solvents. The effects of ethanol and propylene glycol alone were also determined. To determine whether the cardiotonic effect could be nonspecific and related to the physicochemical properties of the fine suspension itself, several experiments were done with varying suspension concentrations of powdered animal charcoal.

A few experiments were performed on the frog heart perfused by the method of Straub to corroborate the results of the preceding method.

The isolated rabbit heart was perfused according to the method of Langendorff, the left ventricle being connected to a light high-frequency lever which recorded on a smoked drum. The coronary flow was measured continuously by a Stephenson (21) recorder or by an electrical drop-counter. In order to arrive at suitable criteria for the hypodynamic state of the isolated rabbit heart, 12 control perfusions were done.

The 32 compounds used in this investigation are shown in Tables I, II, and III. Most of these are unsuitable for mammalian heart perfusions because of low solubility. We were able, however, to obtain some bile acids and small quantities of various water-soluble steroid conjugates sufficient for a few frog and mammalian heart perfusions, and we are especially indebted to Drs. Lozinki and Odell of Frosst & Co. (Montreal) for the synthesis of the sodium succinate salts.

Results

A series of control experiments showed that isolated frog hearts became hypodynamic after continuous perfusion with Ringer's solution for 45 to 60 min. No spontaneous augmentation occurred after the first half hour, and therefore no steroid was perfused until 60 to 75 min. after the beginning of the experiment.

The results on frog hearts are summarized in Tables I, II, and III, which include the number of experiments performed with each compound, the mean percentage augmentation produced by the steroid in various experiments, and

TABLE I

CARDIOTONIC EFFECT OF INSOLUBLE STEROIDS ON FROG HEARTS

(Conc. of infused suspension, 0.01 mgm. per ml.)

No. of experiments	Compound	Mean % augmentation*	S. E. of mean
21	Cholesterol	100	13
12	Cholesterol acetate	70	15
14	Ergosterol	58	6
13	Sitosterol	93	13
14	Stigmasterol	88	15
10	Calciferol	92	12
13	Testosterone	69	10
10	Methyl testosterone	47	10
11	Ethinyl testosterone	69	16
12	Estrone	64	13
11	Estradiol	41	6
9	Pregnenolone	81	12
11	Pregnandione	74	10
10	Desoxycorticosterone	41	6
13	Desoxycorticosterone acetate	31	7

* *See text.*

TABLE II

INSOLUBLE STEROIDS WITH LITTLE ACTIVITY IN FROG HEARTS

(Conc. of infused suspension, 0.01 mgm. per ml.)

No. of experiments	Compounds	Mean % augmentation	S.E. of mean
13	Androstanedione	26	12
10	Androsterone	7	3
11	Dehydroisoandrosterone	22	12
9	Ethinyl estradiol	9	5
13	Progesterone	Depressed	

the standard error of the mean. Percentage augmentation was calculated as the maximum amplitude of contraction (in millimeters) during the steroid perfusion, minus the amplitude during the hypodynamic, presteroid infusion,

TABLE III

CARDIOTONIC EFFECT IN FROG HEARTS OF RELATIVELY SOLUBLE STEROID SALTS AND BILE ACIDS

Compound	Concentration (μgm./ml.)	No. of experiments	Mean % augmentation	S.E. of mean
Sodium cholesteryl succinate	1	10	52	14
Sodium pregnenolone succinate	10	6	34	7
	5	4	46	7
Sodium testosterone succinate	10	9	28	3
	5	4	47	11
Sodium dehydroisoandrosterone succinate	10	7	56	14
	5	3	45	—
Sodium estrone sulphate	10	6	24	14
Desoxycorticosterone β-glucoside	2	11	21	3
Premarin	10	5	61	13
	5	2	55	—
	2	2	60	—
	1	2	30	—
Cholic acid		12	47	9
Desoxycholic acid		11	100	20
Sodium desoxycholate	200	2	5	—
	100	6	19	4
	50	5	18	3
Sodium dehydrocholate	200	12	66	11
	100	7	23	8
	50	3	13	—
Sodium glycocholate	100	4	49	14
	50	4	48	10
	25	4	64	38
	10	3	31	4
	5	5	11	2

divided by the hypodynamic amplitude; this ratio is multiplied by 100. The tables show that 27 of the compounds tested produced marked, though variable, augmentation of hypodynamic frog hearts. The heart rate did not change appreciably, and none of the compounds produced systolic arrest. Table II includes those steroids in which the cardiotonic action was either small or absent. These compounds usually produced bradycardia and initially decreased the amplitude. This depression was often followed by a return to the hypodynamic level, and occasionally even to slight augmentation.

Fig. 1 illustrates the cardiotonic effect of stigmasterol, premarin, alpha-estradiol, and sodium glycocholate in isolated frog hearts. It can readily be seen that the increased force of contraction was not accompanied by changes in heart rate, or any tendency towards systolic arrest. Cardiotonic

PLATE I

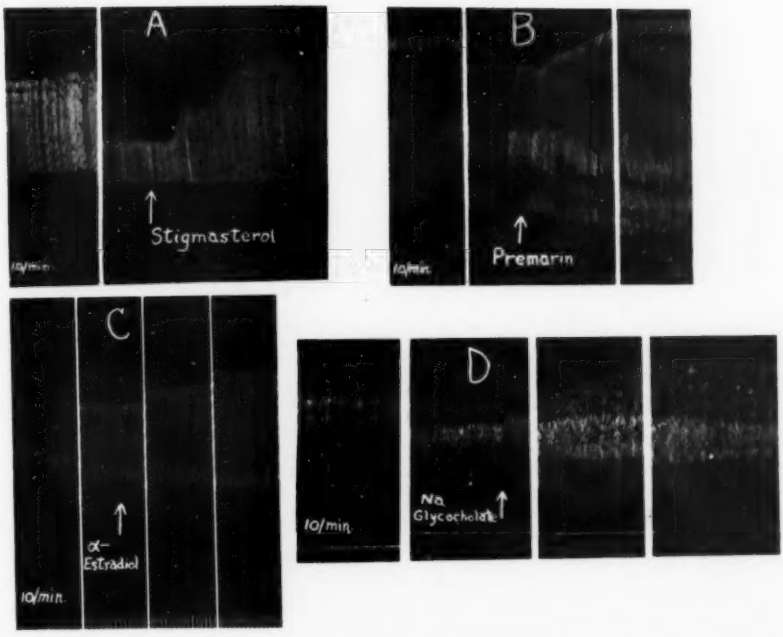

FIG. 1. Isolated frog hearts. A: Stigmasterol, augmentation 188%. B: Premarin (10 μgm. per ml.), augmentation 46%. C: α-Estradiol, augmentation 53%. D: Sodium glycocholate (100 μgm. per ml.), augmentation 70%. Note the absence of changes in heart rate after addition of the drug.

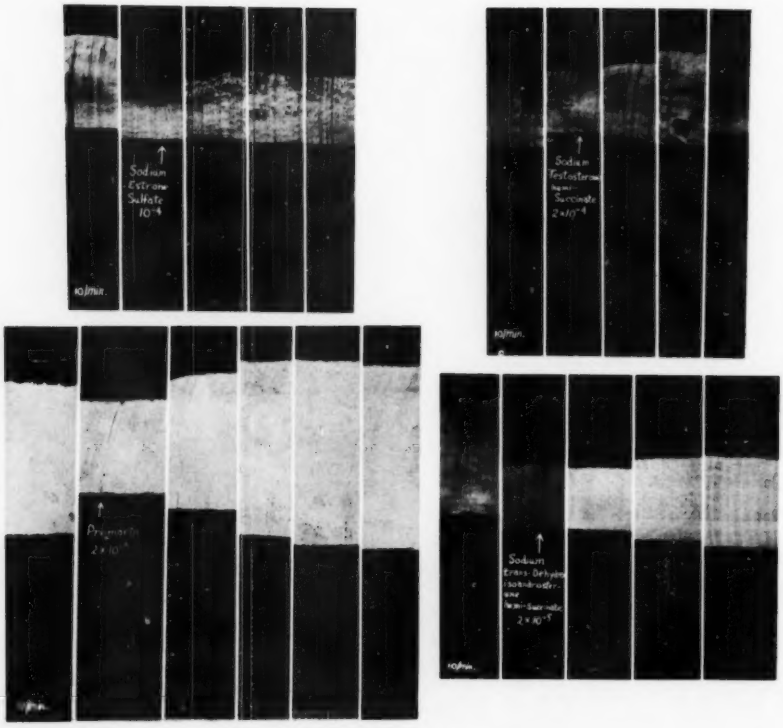

FIG. 2. Isolated rabbit hearts.

	(i)	(ii)	(iii)	(iv)	(v)	(vi)
A: Sodium estrone sulphate (0.1 mgm./per ml.)						
Time	4 : 50	5 : 40	5 : 48	5 : 51	5 : 53	
Heart rate	150	130	144	140	142	
Cor. flow (ml. per min.)	15.3	11.7	21.7	18.3	21.0	
B: Sodium testosterone hemisuccinate, (0.2 mgm. per ml.)						
Time	2 : 20	3 : 00	3 : 07	3 : 10	3 : 15	
Heart rate	150	120	210	240	200	
Cor. flow	10.0	7.0	16.6	12.7	11.7	
C: Premarin (0.02 mgm. per ml.)						
Time	10 : 20	10 : 50	10 : 56	10 : 57	11 : 02	11 : 07
Heart rate	132	128	124	132	142	142
Cor. flow	8.7	6.8	12.0	15.0	14.0	14.3
D: Sodium *trans*-dehydroisoandrosterone hemisuccinate (0.02 mgm. per ml.)						
Time	3 : 15	4 : 00	4 : 05	4 : 10	4 : 15	
Heart rate	200	176	200	236	256	
Cor. flow	33.3	15.3	17.0	18.0	17.3	

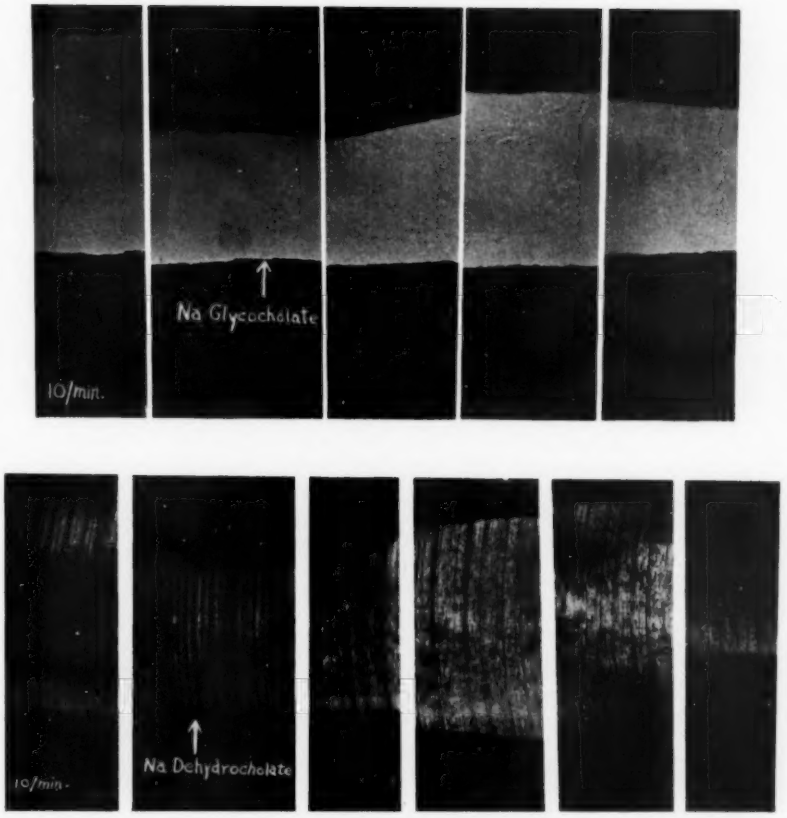

FIG. 3. Isolated rabbit hearts.

	(i)	(ii)	(iii)	(iv)	(v)	(vi)
A: Sodium glycocholate (0.2 mgm. per ml.)						
Time	10 : 10	11 : 50	12 : 00	12 : 05	12 : 10	
Heart rate	116	106	116	110	110	
Cor. flow (ml. per min.)	5.3	5.0	11.4	11.3	9.5	
B: Sodium dehydrocholate (0.2 mgm. per ml.)						
Time	12 : 00	12 : 40	12 : 46	12 : 48	12 : 53	1 : 36
Heart rate	108	102	102	114	104	84
Cor. flow	6.0	6.0	9.0	12.0	16.0	13.0

activity was produced whether the steroid was soluble in Ringer's solution (e.g. premarin—conc. 1 : 100,000 and sodium glycocholate—conc. 1 : 10,000) or whether the steroid was perfused as a suspension (e.g. stigmasterol and α-estradiol).

The study of the effects of steroids on the perfused mammalian heart was necessarily limited by the small number of soluble compounds. The results were variable, and in most cases insufficient quantities of steroids were available to allow a statistical evaluation of their effects. In 12 control experiments it was found that continuous perfusion of isolated rabbit hearts with Locke–Ringer solution for 30 to 90 min. led to a hypodynamic state in which the heart rate, coronary flow, and amplitude of contraction were reduced well below initial levels and were steadily decreasing. No spontaneous augmentation was observed after the first 30 min. of perfusion. The steroids were not perfused until after at least 30 min. of perfusion with Locke–Ringer, at which time the amplitude of contraction had been reduced to 70–50% of the initial level.

Figs. 2 and 3 show the cardiotonic effect of several steroids on isolated perfused rabbit hearts. It can be seen that the maximum increase in the force of contraction of the heart occurred after 10–15 min. perfusion with the steroid. In spite of continuous perfusion of steroid, however, the amplitude

TABLE IV

EFFECTS OF VARIOUS STEROIDS ON ISOLATED RABBIT HEARTS

Compound (conc.)	No. of experiments	Mean % change in heart rate (S.E. shown)	Mean % change in cor. flow	Mean % change in amplitude of contraction
Sodium dehydrocholate (0.2 mgm./ml.)	11	8 ± 1	49 ± 5	4 ± 9.6
Sodium glycocholate (0.2 mgm./ml.)	7	0 ± 2.8	116 ± 51	14 ± 7.8
Premarin (0.2 mgm./ml.)	5	12 ± 3.2	66 ± 22	23 ± 36
Sodium *trans*-dehydroisoandrosterone succinate (0.02 mgm./ml.)	4	16	12	4
Sodium pregnenolone succinate (0.04 mgm./ml.	3	0	11	−11
Sodium testosterone succinate (0.2 mgm./ml.)	3	51	82	−1
Sodium estrone sulphate (0.1 mgm./ml.)	3	7	83	10
Desoxycorticosterone β-glucoside (0.01 mgm./ml.)	2	2	20	−8

gradually decreased again to the hypodynamic presteroid level, and then continued to fall to very low levels as in control experiments. In Table IV a summary of the effects of the soluble steroids on rabbit hearts is given. The concentration of steroid in the perfusion fluid, the number of experiments performed with each compound, and the mean percentage changes in heart rate, coronary flow, and force of contraction are shown. The standard error of the means of the cardiac changes are shown where the number of experiments done warranted their calculation. It can readily be seen that although the changes in heart rate and force of contraction were generally small, the coronary flow usually increased significantly.

Discussion

It can be seen from the results that most of the steroids produced a significant augmentation in the force of contraction of the isolated hypodynamic frog heart with very little change in heart rate. Table I gives an indication of the relative cardiotonic potencies of the steroids. The most active were: cholesterol, desoxycholic acid, sitosterol, calciferol, stigmasterol, and pregnenolone, which all produced average augmentations of 80–100% over the hypodynamic level, and in no case did they fail to produce a positive inotropic effect. No augmentation was observed if the steroids were perfused before the heart had become hypodynamic.

That the increased force of contraction was produced by the steroids themselves is clear from the following results. In no experiment did the force of contraction of the frog hearts spontaneously increase after the hypodynamic level had been reached. The solvents, ethanol and propylene glycol, either had no effect, or actually depressed the hearts. In several experiments the pure crystalline steroid was dropped into the perfusion cannula; the resulting suspension regularly caused augmentation of the heart beat. Evidence that the effect was specific and not simply a question of the physicochemical properties of the perfused suspension was obtained by perfusing an insoluble, nonsteroidal substance, animal charcoal. No augmentation was observed. Also, cardiotonic activity was found with soluble as well as insoluble steroids, and with the soluble conjugates of insoluble steroids. Thus testosterone invariably produced an increased force of contraction whether it was first dissolved in ethanol, or in propylene glycol, added as a water suspension of "microcrystals", or merely dropped into the cannula as ordinary testosterone crystals, or perfused as the soluble conjugate, sodium testosterone succinate.

The results of the method of perfusing the frog hearts through the vena cava were checked by another perfusion technique—that of Straub. Positive inotropic activity was produced by both methods in the same concentration range of the steroids. The vena cava perfusion technique was used throughout this investigation because changes in heart rate are more valid with this method than in the Straub heart where the sinus tissue is not in direct contact with the perfusion fluid.

In the perfused mammalian hearts the results show that the coronary flow generally increased whether the heart rate and force of contraction changed or not. For this reason it is thought that where augmentation does occur at least part of the augmentation is due to a direct "tonic" effect of the steroid on the heart muscle, and is not due entirely to the increased coronary flow.

None of the steroids shown in the tables produced systolic arrest. However, when digitonin was tested, systolic standstill was often observed. Although Sollmann stated (20) that digitonin was not cardiotonic in the frog heart, our results with the other steroid compounds prompted us to test its activity again. It was found that digitonin in concentrations of 1–2 μgm. per ml. also produced augmentation, and the augmentation was accompanied by systolic arrest. Digitonin differs from the other steroids investigated in that it contains two lactone rings attached to the cyclopentenophenanthrene nucleus. The classical action of the digitalis glucosides is augmentation followed by systolic standstill, and this was observed in several rabbit hearts perfused with Lanatoside C. These compounds contain both the steroid nucleus and a lactone ring. Thus the importance of the lactone ring in the production of systolic arrest is evident. We find that the steroids themselves produce marked augmentation without systolic arrest, but when the steroid is coupled with a lactone ring as in digitonin or Lanatoside C the augmentation is followed by systolic standstill. Confirmation of the idea that the lactone ring is responsible for systolic arrest is found in recent work (3, 8, 13) which showed that certain lactones themselves produced systolic arrest.

In spite of the vast amount of research done with the cardiac glycosides, their exact mode of action is not yet understood. Therefore it is even more difficult to explain why the steroids are cardiotonic. That they may act similarly to the glycosides is quite possible, since a recent paper by Govier (9) suggests that the cardiac glycosides and several steroids, cholesterol, digitonin, and certain sex hormones increase lactate oxidation by stimulating the anaerobic lactic dehydrogenase system in cardiac-muscle homogenates. On the other hand the lactones which produce augmentation and systolic arrest are metabolically inactive (28). It thus appears that the steroid structure of the cardiac glycosides is very important for their cardiotonic action.

Acknowledgments

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L'ACTION DU JEÛNE SUR LE DÉVELOPPEMENT DE L'ARTHRITE EXPÉRIMENTALE[1]

PAR PIERRE DUCOMMUN[2] ET LUCIEN L. COUTU

Résumé

Les auteurs ont étudié l'action du jeûne sur l'arthrite expérimentale chez le rat intact et surrénalectomisé. Le jeûne prévient l'apparition des phénomènes inflammatoires chez les rats intacts et privés de surrénales. La reprise du régime normal est suivie de l'apparition des manifestations inflammatoires locales chez les rats intacts et surrénalectomisés. Ces faits suggèrent que les glucocorticoides ne sont pas seuls responsables de la prévention de l'arthrite expérimentale lors du jeûne.

On sait que le jeûne diminue les manifestations inflammatoires produites par un agent irritant introduit sous la peau: Selye puis Coutu (1, 3) l'ont bien démontré avec l'arthrite à la formaline et l'arthrite à la moutarde. Cet effet antiphlogistique a été attribué par ces auteurs, à une décharge de glucocorticoides sécrétés par la surrénale sous l'influence de l'augmentation de l'ACTH endogène au cours de l'état de stress. Dans ce cas, le jeûne agit comme agent stressant.

Des observations préliminaires chez le rat intact et au jeûne nous ont permis de constater que l'arthrite se manifeste au lieu d'injection de l'agent irritant lorsque les animaux sont remis à leur régime habituel. Cette constatation nous a poussé à étudier ce phénomène chez les rats surrénalectomisés afin de vérifier si la privation de nourriture agissait sur le tissu enflammé par l'état de stress qu'elle déterminait.

Matériel et Méthodes

Quarante rats de la race Sprague Dawley de 180 à 200 g. ont été divisés en quatre groupes. Les animaux des deux premiers groupes ont été surrénalectomisés quatre jours avant le début de l'expérience. Tous les animaux ont été maintenus à un régime composé de Purina Fox Chow et d'eau additionnée de 1% de NaCl et de 5% de glucose, six jours avant le début de l'expérience. Tous les rats ont été mis au jeûne en maintenant la boisson (eau additionnée de 1% de NaCl) 48 hr. avant la création d'une arthrite par injection de 0.2 cc. d'une suspension de moutarde Keen, dans la région tibiotarsienne de la patte gauche (test de Coutu) (1).

Les animaux du groupe I ont été remis au régime normal deux jours après l'induction de l'arthrite, ceux du groupe III, trois jours après. Les rats des groupes I et IV ont été maintenus au jeûne jusqu'à la fin de l'expérience.

Les mesures de l'articulation irritée ont été faites chaque jour et la tension artérielle a été mesurée dans les groupes I et II, le quatrième jour du jeûne.

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Contribution de l'Institut de Médecine et de Chirurgie Expérimentales, Université de Montréal, Montréal, Québec.

[2] *Boursier de l'Académie Suisse de Médecine.*

Résultats

Nos résultats sont rassemblés dans le tableau suivant.

TABLEAU I

INDEX DE RÉACTIVITÉ AU TEST DE L'ARTHRITE

Traitement	Réaction aiguë	Jour *				Pression artérielle
		1er	2e	3e	4e	
Surrénalectomie	3.0	2.2	*2.5*	*3.6*	*4.6*	115
Surrénalectomie	2.9	2.2	2.4	2.6	—	85
Intact	2.7	2.2	2.8	*3.8*	*4.4*	
Intact	3.0	2.1	2.2	2.9	2.8	

* *En italique = au jeûne. En caractère droit = régime normal.*

Comme on peut le voir, à la lecture du tableau précédent, trois faits principaux peuvent être mis en évidence.

(1) Le jeûne diminue les manifestations inflammatoires de l'arthrite chez le rat intact et chez le rat surrénalectomisé.

(2) La reprise du régime normal est suivie de l'apparition des phénomènes inflammatoires tant chez le rat intact que chez le rat surrénalectomisé.

(3) La tension artérielle est légèrement abaissée chez le rat surrénalectomisé à jeûn.

Discussion et Conclusions

Comme nous l'avons rappelé dans l'introduction de nos expériences, Selye et Coutu (1, 3) ont déja constaté l'action "anti inflammatoire" du jeûne et l'ont rapportée à l'action stressante de la privation de nourriture.

Nos résultats confirment leurs observations et mettent en évidence le fait que les glucocorticoides ne sont pas seuls responsables de la diminution des phénomènes inflammatoires dans ces conditions. En effet, si le jeûne agissait uniquement par l'intermédiaire de l'axe hypophysosurrénalien, la surrénalectomie abolirait complètement cette action "anti phlogistique". Au contraire, elle persiste chez les rats privés de surrénales et est de même intensité que chez les animaux intacts.

La reprise d'un régime normal est suivie de l'apparition de phénomènes inflammatoires au lieu d'injection de la moutarde, tant chez les rats intacts que chez les surrénalectomisés. Ce fait est une indication de plus que le jeûne n'agit pas uniquement par l'intermédiaire de l'axe hypophysosurrénalien.

La mesure de la pression artérielle a été effectuée pour tenter d'approcher le mécanisme d'action du jeûne. En effet, Hirschfelder (2) a constaté que la réaction œdémateuse oculaire due à l'irritation causée par de l'huile de

moutarde est diminuée par la ligature des carotides. Selye (4) a démontré que la ligature de l'artère fémorale prévient temporairement l'apparition des phénomènes inflammatoires dus à l'injection d'un irritant (kaolin) dans l'articulation tibiotarsienne du rat.

Il est possible que la prévention observée dans nos expériences soit due à la diminution de la tension artérielle et partant au manque d'apport de matériel nécessaire à l'élaboration du granulome. Coutu a en effet démontré que la réaction arthritique est diminuée sous l'influence d'un régime alimentaire pauvre en protéines. Cependant, quelles que soient les explications recherchées pour élucider ce mécanisme d'action, il semble très peu probable, à la lumière des faits que nous rapportons, que les glucocorticoides surrénaliens soient seuls responsables de la diminution des phénomènes inflammatoires observés sous l'influence du jeûne.

Remerciements

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THE OCCURRENCE OF ZINC IN THE HUMAN PROSTATE GLAND[1]

By C. A. Mawson and M. I. Fischer

Abstract

The prostate gland contains a higher concentration of zinc than any soft tissue in the human body previously investigated. In an extensive series of analyses Eggleton found the highest concentration in liver (245 μgm. Zn per gm. dry weight), whereas the mean zinc content of all our specimens of human prostate was 682 ± 70 μgm. per gm. dry weight. Carcinomatous prostates contained much less zinc than normal glands, and the amount of zinc in any given prostate was directly related to the proportion of alveolar tissue present.

The dorsolateral (or posterior) prostate of the rat has been shown by Mawson and Fischer (7) to contain very much more zinc per unit weight of ash than any other rat tissue examined. Rabbit prostate also contains very large amounts of zinc, so it was of interest to investigate human prostate, particularly as zinc has sometimes been thought to have significance in relation to cancer. The nuclei of cells have been shown by Miyake (8) and Koga (6) to be richer in zinc than the cytoplasm, and Addink (1) has suggested that the liver and blood of cancer patients may contain more zinc than those of persons without malignant disease. Radioactive zinc was injected by Heath (4) into mice bearing a leg sarcoma and a mammary carcinoma. He found, in agreement with Rosenfeld and Tobias (9), that only a small amount of zinc remained in the nuclei of liver, kidney, spleen, and tumor after extraction with citric acid, but the desoxyribosenucleoprotein separated from nuclei which had not been treated with acid contained about one-third of the radioactivity of the whole tissue. It seems likely that zinc is concerned in some way with the nucleus, but little more than this can be said at the present time.

Material and Methods

Specimens of human prostate were received in zinc-free containers. About half the material consisted of curettings taken during transurethral prostatectomy, and although these usually arrived in fairly fresh condition it was impossible to say from what portion of the gland any one fragment had been taken. The remainder of the specimens came from the autopsy room at the Department of Pathology, McGill University, as slices about 0.5 to 1 cm. thick, and many of these were somewhat decomposed. Pieces of tissue weighing about 200 mgm. were used for the estimation of zinc, three to five replicates being taken in most cases. Wherever possible adjacent pieces were fixed in absolute alcohol for histological examination.

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Zinc was estimated by the method of Vallee and Gibson (10) after ashing at 580° C. Tissue sections were stained with haematoxylin and eosin and also with Okomoto's specific stain for zinc, as described by Kadota (5).

Results

Zinc Estimations

The zinc content of the human prostate was found to be very high, in agreement with Bertrand and Vladesco (2), who reported results of 489 and 533 μgm. Zn per gm. dry weight in two glands and remarked that these were the highest figures they had obtained from any human tissue.

Our results (Table I) have been divided into groups according to the diagnoses received from the pathologists who supplied the specimens. Owing to the nature of the sources of our material no glands were received from

TABLE I

ZINC CONTENT OF HUMAN PROSTATE GLAND

Condition	"No lesions"	Hypertrophy	Carcinoma	Chronic prostatitis	No diagnosis	All analyses
Mean zinc content (μgm./gm. dry weight)	859	772	190	332	613	682
Range	598-1265	268-1806	65-399	—	261-1281	65-1806
Standard error	96	93	61	—	—	70
Number of glands	7	20	5	1	3	36

normal individuals, and the figures listed under "No lesions" represent tissue from patients who had died from causes which did not involve the prostate. The zinc content of the glands in this group did not differ significantly from that of the hypertrophy group. Very low results were obtained from the five carcinomatous prostates examined, and this is of interest as malignancy has previously been associated with high zinc concentrations. Statistical analysis shows that the difference between the "No lesions" and the carcinoma groups is highly significant ($P < 0.01$).

When the tissues were arranged (Table II), irrespective of diagnosis, according to the number of alveoli observed histologically in the immediately adjoining region, the zinc content of tissues containing many alveoli was found to be significantly greater than that of tissues containing few alveoli.

Specific Staining for Zinc

The results with Okomoto's stain were disappointing. Most of the positive results obtained with human prostate were located in fissures, along cut edges, and at apparent sites of autolysis. Occasionally groups of alveoli stained more deeply than the surrounding muscle and connective tissue, but the differences were never comparable with the striking contrasts seen in rat dorsolateral prostate.

TABLE II

ZINC CONTENT OF HUMAN PROSTATE GLANDS CONTAINING DIFFERING PROPORTIONS OF SECRETORY TISSUE

Relative numbers of alveoli present	Few	Moderate	Many
Mean Zn content (μgm./gm. dry weight)	621	793	1176
Standard error	95	98	154
Number of specimens	14	13	11

NOTE: *Differences are significant between Columns 2 and 3 ($P < 0.05$) and columns 1 and 3 ($P < 0.01$).*

Discussion

The prostate glands of the rat and the rabbit contain much more zinc than any other soft tissue examined, with the exception of the leucocytes. The mean value for the dorsolateral prostate of the rat (with standard error) was 874 ± 68 μgm. per gm. dry weight, and for the rabbit prostate 1296 ± 81 μgm. per gm. The human material at our disposal consisted largely (60%) of hypertrophied glands, and although many contained an abnormally large amount of nonsecretory tissue, the mean zinc content of all the human prostates examined was 682 ± 70 μgm. per gm. dry weight. This is considerably higher than the highest mean value found for zinc in normal human tissues by Eggleton (3). His highest values were for liver (245 μgm. per gm.) and muscle (266 μgm. per gm.) and the lowest were for cerebrum (43 μgm. per gm.) and testis (62 μgm. per gm.). He did not examine the prostate.

The number of specimens of carcinoma received was small, but there was evidence of a decreased zinc content in glands containing malignant tissue. There was also good evidence that a high zinc content was associated with a high proportion of organized secretory tissue. The significance of zinc in the prostate is unknown, but the large amount of this element found in the prostates of three different species suggests that it may have some physiological importance.

It is hoped that our results with human material may stimulate the interest of workers who have easier access to suitable material than is the case in this laboratory.

Acknowledgments

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THE EFFECT OF TEMPERATURE ON THE CIRCULATION OF THE ISOLATED PERFUSED RABBIT EAR[1]

By James T. Nichol[2]

Abstract

Isolated rabbit ears were perfused with Ringer solution, and the rates of flow were measured by a micro flow-gauge. It was found that changes in flow produced by changes in temperature of the ear (amounting to two and a half times between 40° and 0° C.), could be quantitatively explained by changes in the viscosity of the perfusion solution with temperature. No evidence of a "hunting reaction" to cold could be demonstrated either with or without a temperature gradient in the ear. The residual "critical closing pressure" of the isolated rabbit ear was shown to be independent of temperature. It is concluded that in the isolated perfused rabbit ear the temperature effects on circulation are entirely explicable in terms of viscosity changes, and that in the intact innervated ear, viscosity effects also underlie, but do not completely explain the observed changes with temperature.

I. Introduction

The vascular bed of the rabbit ear is a particularly interesting one in that it possesses, in addition to capillaries, numerous arteriovenous anastomoses of much larger caliber than that of capillaries; the existence of these shunts is believed to be associated with the temperature regulating properties of the ear.

The first mammalian arteriovenous anastomoses were described in the fingers and toes of man by Sucquet in 1862 (25), a fact which has been confirmed subsequently by many investigators. Since the fingers and toes in man appear to assist in temperature regulation by virtue of their wide range of heat loss through radiation, the belief developed that the anastomoses aided in this function (19, 20). Grant first studied arteriovenous anastomoses in the living animal by subjecting carefully prepared rabbits' ears to direct microscopic observation by means of strong transmitted light (13). Grant and Bland later studied these anastomoses in human skin and the bird's foot (14). Clark and Clark studied the structure and formation of the anastomoses by means of a transparent chamber in the rabbit ear (9).

The effects of temperature on flow in a peripheral temperature regulation region such as the human finger or the rabbit ear have been studied by many investigators. It has been noted generally that the application of heat produces a dilation (2). On the application of cold there is an initial vasoconstriction as a result of the direct action of the stimulus on the blood vessels; after this has continued for a short time a vasodilation takes place (1). The means by which temperature effects change in flow has been considered by many persons. Abramson notes that the vascular shunts are richly provided with sympathetic nerves and can readily dilate or constrict (2). In addition

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[2] *Holder of Fellowship from the National Research Council of Canada.*

to the sympathetic control over the shunts, there is also the question of nervous control over the vascular bed as a whole. Clark and Clark have noted a rhythmic contraction which extends over all the arteries and arterioles of the rabbit ear (8), and the rate of contraction noted by these observers was about one to three times per minute. Grant noted that these rhythmic contractions were influenced by the central nervous system (12). This phenomenon was found in the human finger by Burton (5). It has been suggested that some of the waves of contraction in the finger are due to local activity of the smooth muscle, independent of the innervation, while the majority of them seem to be dependent upon vasomotor activity (16).

Oscillations in temperature of much longer duration (several minutes) have been noted by Grant in the rabbit ear (13) and in the human finger by Lewis (21). This was termed a "hunting reaction". Lewis noted that this oscillation occurred immediately after the mixed nerve was cut but did not occur after degeneration of the whole nerve supply. Degeneration of the sympathetic nerve supply had no effect on the oscillations. He believed that the dilatory phase of the oscillation resulted from a sensory axone reflex produced by the liberation of a histamine-like substance during the constrictive phase. The "hunting reaction" has been later described by Wolff and Pochin (27). More recently Greenfield and Shepherd have studied the circulation of the finger at low temperatures (15). They have obtained the same "hunting reaction" as Lewis did in 1930. They have noted as well that the nature of the response was characteristic of the individual and that simultaneous measurements on the fingers of both hands revealed that the oscillations in flow could get out of phase. Greenfield and Shepherd state that they are uncertain as to what extent changes in blood viscosity in the cold finger may have been responsible for changes in blood flow which they have interpreted as changes in vessel caliber.

Since the reactions of vascular beds, to temperature, particularly those containing arteriovenous shunts, may involve so many different factors, local and general, humoral and nervous, it is obvious that any analysis of these factors must start with a knowledge of the underlying purely physical factor of increased viscosity at lower temperatures. Accordingly the present study was undertaken with the primary purpose of finding to what extent changes in the viscosity can explain the effects of temperature on the circulation of the excised rabbit's ear. During the course of other work in our laboratory, it has been found that vasomotor tone due to the excision may exist for the first hour, but after this the vessels are dilated and the responsiveness to injection of adrenalin remains good and very constant for many hours.

II. Methods and Apparatus

The rabbit ear was excised and prepared for perfusion with a buffered Ringer solution. The method and composition of the solution have been described (22). The Ringer solution was allowed to flow from a reservoir at a measured height above the ear, through a microflowmeter which has been

described (7), and then through a coiled glass tube into a cannula into the artery in the rabbit's ear. The ear and the coiled glass tube, which was bent in a circle around it, was immersed in water contained by a glass tray, 17 × 15 × 2.5 cm. The temperature of the water in the tray could be controlled. This was done by permitting a continuous flow from a mixing chamber into the tray. In this way the temperature of the ear as well as the perfusion solution which passed into it were brought to the same values. The arrangement of this apparatus is shown in Fig. 1.

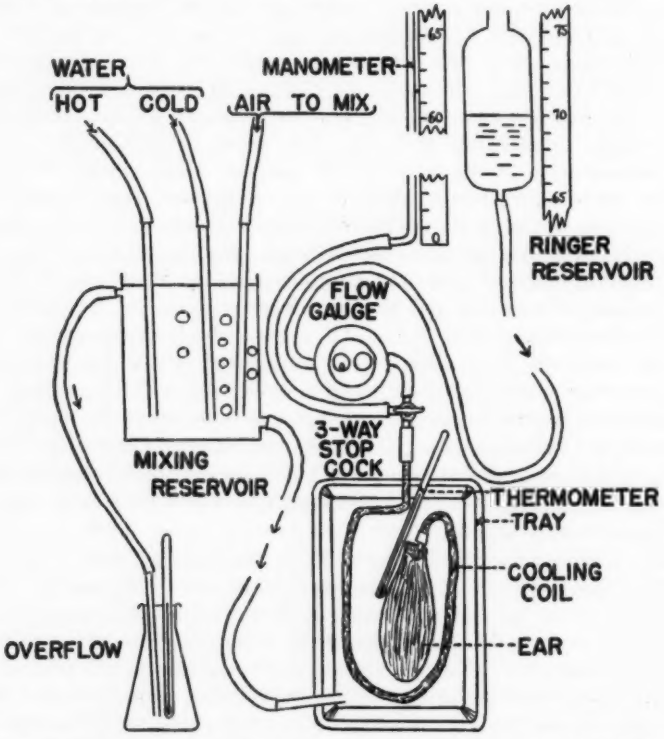

FIG. 1. Component parts of the apparatus used in the perfusion of the isolated rabbit ear.

The resistance of the perfusion apparatus up to and including the cannula was easily determined by measuring the flow through the apparatus with the cannula immersed in the water in the tray, and by dividing the pressure value by the corresponding flow value. Since the coiled glass tube and the cannula were to be used at a variety of temperatures, a series of resistance values with these portions of the apparatus at different temperatures were made.

In earlier experiments in our laboratory we have found that perfusion of the rabbit ear with 1 : 10,000 sodium cyanide for 20 min. is sufficient to abolish

the effects of adrenalin upon the vascular musculature. This method was employed whenever the elimination of vasomotor effects was necessary. Whenever sodium cyanide was so used its efficacy was verified by subsequent perfusion with adrenalin.

III. Results

(1) Effects of Temperature on Resistance of Perfusion Apparatus to Flow

Fig. 2 shows the curve of resistance of the apparatus against the temperature of the perfusing fluid. The resistance at 0° C. is more than twice what it is at 40° C. This is entirely explained in terms of the increase in relative

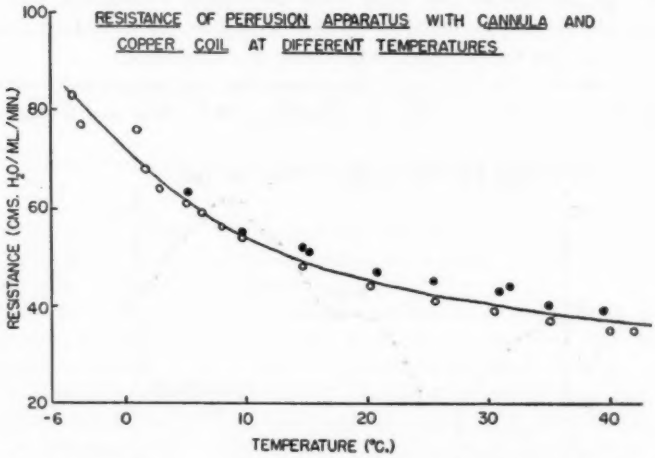

FIG. 2. The effect of temperature upon the resistance to flow of Ringer solution through the perfusion apparatus.

viscosity of the perfusing fluid, which is the same as that of water, as indeed is the relative change in viscosity of blood with temperature. The change in resistance of the apparatus between 0° and 40° C. was not quite as great as the change in viscosity of water between these two temperatures. This is explained by the fact that a small part of the resistance of the apparatus was in the flowmeter and tubing, the temperature of which was not changed, while the large part of the resistance was in the cannula and tubing in the tray. The resistance, at constant temperature, was independent of rate of flow.

(2) Preliminary Studies of the Effect of Temperature on Flow

Flow was measured with the flow gauge when buffered Ringer solution was perfused through the ear. The temperature of the water in the tray surrounding the ear was measured to the nearest tenth of a degree simultaneously with flow measurements. In this series of experiments, the level of the Ringer

solution in the reservoir of the perfusion apparatus was kept at a height of 70 cm. above the level of the water in the tray containing the rabbit ear.

By the use of the mixing reservoir, the temperature of the ear was permitted to rise slowly from 15° to 38° C. Then the temperature was lowered slowly to the freezing point. The later stage of temperature lowering was accomplished by surrounding the ear with sodium chloride and cracked ice. As soon as the cessation of flow indicated that the perfusion solution had begun to freeze, the temperature of the ear was allowed to rise to 15° C. Following this the responsiveness of the ear to adrenalin chloride was measured.

Five ears in all were used to study the simultaneous changes of temperature and flow. In the case of two ears, after the regular experiment had been carried out, the ear was perfused for 20 min. with a 1 : 10,000 sodium cyanide solution. Following this a repeat experiment was carried out.

In all experiments, the relationships between flow and temperature were the same. In Fig. 3 is shown a typical experiment where flow and temperature

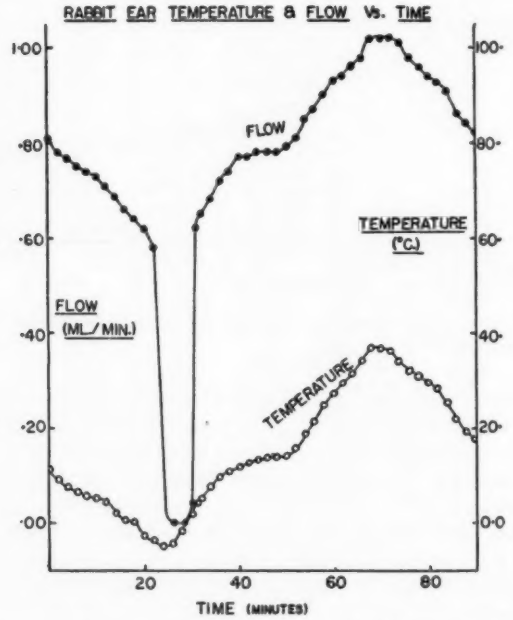

FIG. 3. Changes in flow through the rabbit ear produced by gradually changing the temperature of the vascular bed.

are plotted against time. The remarkable degree of similarity between the curves is notable. The large dip in the center of the flow curve is believed to be due to the freezing of the perfused solution.

In Fig. 4 is shown the relationship between temperature and flow in the same experiment. The lines in the graph do not retrace each other, but the greatest differences in flow do not amount to much more than 15% of the

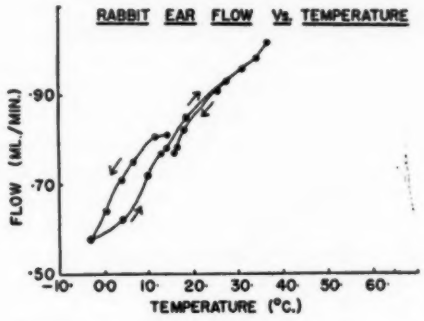

FIG. 4. Changes in flow with changes in temperature of the rabbit ear.

total value. The repeat temperature-versus-flow experiments, which were carried out after the ear had been perfused with sodium cyanide gave results identical with those initially obtained.

The decrease in flow, under constant perfusion pressure, with a fall in temperature, which is observed in the rabbit ear, signifies that there has been an increase in its resistance to flow. Poiseuille's formula, which applies only to undistensible tubes, predicts that, if the vascular bed behaves as a system of undistensible tubes, the resistance will vary directly as the viscosity of the perfusion solution. The viscosity of a liquid, however, is a function of the temperature and increases as this falls. If, therefore, the rate of increase of resistance of the rabbit ear with decreasing temperature could be accounted for by changes in the viscosity of perfusion solution alone, we could conclude that vessels are behaving as undistensible tubes.

Let us suppose that the dimensions and geometry of the vascular bed remain constant in the rabbit ear and that the resistance of the bed at 30° C. is 100 units; then the resistance can be predicted for lower temperatures by the use of viscosity tables for water (18). The viscosity of the Ringer solution is practically the same as that of water. This predicted curve of resistance for the rabbit ear is plotted as the solid line in Fig. 5.

The actual resistances of the ears at different temperatures were determined as follows:

The total resistance of the perfusion apparatus and the rabbit ear to flow may be found by substituting the flow value for a particular temperature, as shown in Fig. 4 in the equation $R = P/F$ (since P is always 70 cm. of water). The resistance so found will be expressed in centimeters of water per milliliter per minute. The resistance of the ear alone was found by subtracting the apparatus resistance at that temperature in Fig. 2 from the total resistance.

The true resistances of the individual rabbit ears obtained by these calculations were not directly comparable with one another or with the predicted curve shown in Fig. 5 since the dimensions of the vascular bed of each ear

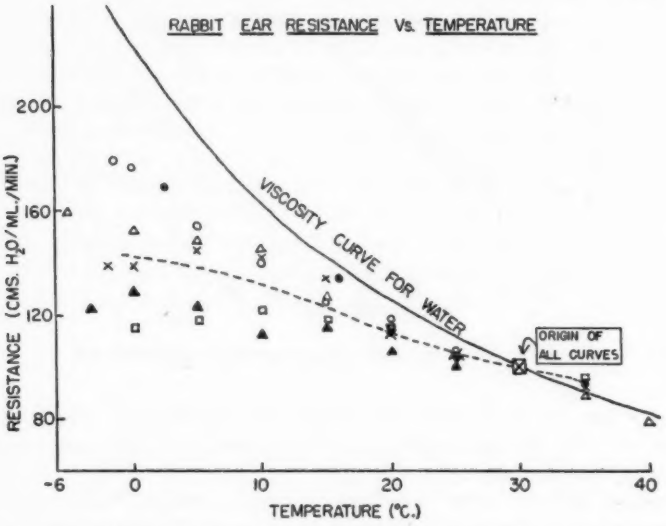

Fig. 5. Resistance of the rabbit ear to flow of perfusion solution versus temperature.

were different. In order that each ear resistance curve could be compared with the predicted curve, they were all made to pass through one point on it (at resistance = 100 units, temperature = 30° C.) this was done by multiplying all the values in the real ear resistance by a factor,

$$\frac{100}{\text{actual resistance of the ear at } 30^\circ \text{ C.}}.$$

These relative resistance values are plotted in Fig. 5. A different symbol is used for each ear. The arithmetical means of the points at a particular temperature have been joined together by the plotted line.

In the ears studied, the resistance to flow does not rise as rapidly with a drop in temperature as it would in the case of rigid tubes. This means that a distension of the vascular bed has occurred. Such a distension might be an active response of the vessels to temperature changes or a passive one due to changes in the pressure in the vessels. When the temperature falls and the resistance increases, flow through the apparatus and the ear decreases, and the active pressure applied at the ear increases since the drop of pressure in the apparatus is proportional to the flow. The actual applied pressure can be determined from the data at hand, but whether the distension of the vessels is due to pressure increase or to the temperature decrease cannot be ascertained because one is always accompanied by the other.

A set of experiments was then designed to determine whether the changes in the resistance in the ear were due to temperature or pressure. They were set up to produce "isobaric" curves of resistance versus temperature.

(3) Isobaric Studies of the Effects of Temperature on Resistance to Flow

Five rabbit ears were excised and the apparatus shown in Fig. 1 was used. Flow measurements with buffered Ringer solution were made with the flow gauge at a number of temperatures: in order 35° C., 30° C., 25° C., 20° C., 15° C., 10° C., 5° C., and then repeats at 15° C. and 30° C. In each case, the ear was kept to within 0.3° C. of the particular temperature. At each temperature, flow was measured in order at a number of total applied pressures: 70, 55, 40, 25, 15, 25, 40, 55, and 70 cm. of water. Each flow was taken two minutes after the pressure had been applied.

The method of treating the results may be most easily explained by the numerical example shown in Table I. Results on the other four rabbits were

TABLE I

TREATMENT OF RESULTS (ISOBARIC STUDIES OF R AND T). TEMPERATURE, 15° C.

Total applied pressure (cm. H_2O)	Flow with decreasing pressure (ml./min.)	Flow with increasing pressure (ml./min.)	Average flow (ml./min.)	Total resistance (cm. H_2O) (ml./min.)	Apparatus resistance at this temperature (cm. H_2O) (ml./min.)	Ear resistance (cm. H_2O) (ml./min.)	Pressure applied at ear (cm. H_2O)
70.0	0.76	0.77	0.76	92.0	48.0	44.0	33.4
55.0	0.56	0.54	0.55	100.0	48.0	52.0	28.6
40.0	0.35	0.31	0.33	121.0	48.0	73.0	24.1
25.0	0.14	0.12	0.13	192.0	48.0	144.0	18.7
15.0	0.07	0.07	0.07	214.0	48.0	166.0	11.6

comparable. The two flow values were averaged. This average flow value was divided into the total applied pressure value to give the total resistance. From this, the resistance of the perfusion apparatus including the cannula (obtained from Fig. 2) was subtracted to give the resistance of the ear alone. As soon as the resistance of the ear was known, the pressure applied could be found by multiplying the resistance value by the flow value.

The next step was to plot the pressure applied at the artery against the resistance of the ear. By this means a number of isothermal curves were produced. Lines were then drawn parallel to the resistance axis to intersect the pressure axis at pressure values of 15, 20, 25, and 30 cm. of water. The points where these lines cut the isothermal curves have been used to make the isobaric plot shown in Fig. 6.

The lines shown in Fig. 6 have not been drawn from the points of the graph. Rather a single resistance value has been arbitrarily selected for the particular pressure at 35° C. This point has been picked so as to be as close as possible

to the best line that could be drawn for the experimental points. The resistance-versus-temperature curves have been extended from these points purely on the basis of increments in viscosity which could be expected to

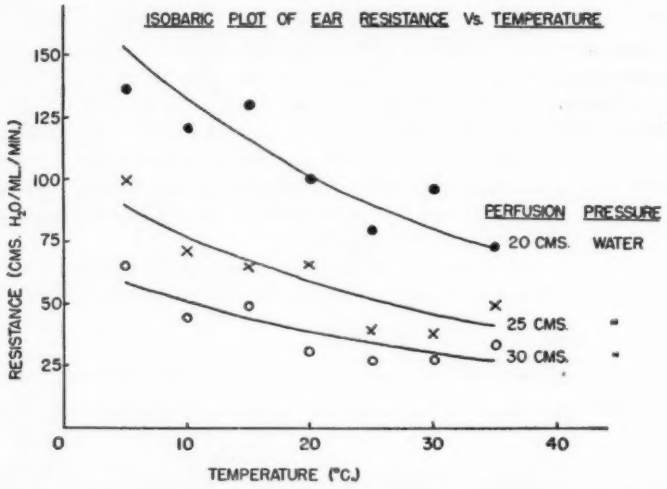

FIG. 6. Isobaric plots of rabbit ear resistance to flow of perfusion solution versus temperature. Derived by an analysis of curves such as Fig. 5 for different perfusion pressures and temperatures.

occur as the temperature of water is lowered while it is being perfused through an inextensible tube. It can be seen that these curves are, for all practical purposes, those which the experimental points would lead us to construct. Fig. 6 thus indicates that the vascular bed of the rabbit ear does not change its dimensions with change of temperature if the pressure is kept constant. At the end of each of these experiments the viability of the ear was checked by perfusion of the ear with 1 : 1,000,000 adrenalin chloride in Ringer solution. In every case a 90% of more reduction in flow resulted. In cases where the responsiveness of the ear was destroyed by sodium cyanide perfusion (1 : 10,000) no changes in the curve were produced. Viability of the smooth muscle is, then, not a factor. Fig. 6 indicates that the increase in resistance can be explained purely on the basis of the viscosity of the buffered Ringer solution at different temperature.

(4) Prolonged Perfusion at Low Temperatures

A series of experiments were carried out to ascertain whether the oscillatory flows which have been reported by Lewis in the human finger (21) could be produced in the isolated rabbit ear.

In the first set of experiments the same apparatus described above was utilized. Ringer solution at a pressure of 88 centimeters of water was perfused for 45 min. through the ears at 18 $\pm$ 1° C. and then for a similar length

of time at 8 ± 1° C. No "hunting reaction" of any sort was obtained. Fluctuations in flow did not exceed 5% and could be accounted for by slight changes in viscosity due to slight temperature fluctuations.

In a second set of experiments the method was altered in that the Ringer solution instead of being precooled to the temperature of the ear by the cooling coil was, rather, heated to 37° C. while the ear was kept in cold water. The cannula and a very small area of the ear adjacent to it were held out of the tray so that no precooling of the perfusion solution would occur. Perfusions of 45 min. duration were made first with the tray at 21 ± 1° C. In no case was any "hunting reaction" observed, even when this gradient of temperature existed.

At the conclusion of each of these experiments the viability of the preparation was tested by perfusion with 1 : 100,000 adrenalin chloride. In every case a percentage reduction in flow up to 90% was secured.

(5) *Effect of Temperature on "Critical Closing Pressure"*

The phenomenon of "critical closing pressure" has been described elsewhere in some detail (6, 24). This critical closing pressure was measured in the case of several cooled rabbit ears. The vertical tube method which has been described elsewhere was utilized in securing these values (23). The critical closing pressure obtained with Ringer solution alone was the usual one obtained for the relaxed ear at higher temperatures, i.e. 3 to 4 cm. of water. A typical set of results is shown in the tabulation below:

CRITICAL CLOSING PRESSURE VERSUS TEMPERATURE

Temperature, °C.	5	10	15	20	25	30	35
Critical pressure (cm. water)	3.20	3.15	3.45 3.65	3.25	3.25	3.30	3.80 3.40

IV. Discussion

The considerable changes in viscosity produced by small changes in temperature have seldom been appreciated by biologists, who have been rather prone to attribute all flow effects to various neuromuscular mechanisms. Although these mechanisms are undoubtedly important it is unjustifiable to ignore viscosity.

We have shown that all changes in the flow of Ringer solution through the isolated rabbit ear can be accounted for by changes in viscosity, or by changes in the dimensions of the vascular bed due to simple elastic stretch. If the latter factor is eliminated by perfusing at a constant applied pressure at different temperatures, flow changes are quantitatively explainable by viscosity changes.

Now it may be argued that it is dangerous to apply these findings with such a simple substance as Ringer solution to the normal rabbit ear containing blood. Blood has been considered by many to have an anomalous viscosity, such viscosity varying not only with the rate of flow of blood but also with the

diameter of the tubes through which it is flowing (10, 17). However these anomalous viscosities have been obtained with the use of glass tubes and as such mean little from a physiological point of view. The only fair comparison between the viscous behavior of Ringer solution and blood should be made in a vascular bed. This has been done by Whittaker and Winton in the perfused hind limb of the dog (26). These observers found that the viscosity of blood was 2.2 ± 0.2 times that of Ringer solution under similar conditions, but increased slightly in value at applied pressures below 50 mm. Hg. More recently work has been done in our laboratory where Ringer solution and blood were perfused through the same rabbit leg (23). Here the viscosity of blood appears to be about twice that of the Ringer solution as long as the applied pressure is sufficiently above the critical closing value. The whole problem of rheology of the blood has been reviewed in some detail by Bingham and Roepke (3, 4). They have pointed out that it is possible to predict the fluidity of blood at any temperature by means of a few easily determined constants.

It is logical therefore to conclude that, in life, viscosity changes of the blood in the rabbit ear with changes in temperature will account for changes in flow which will be proportional to those changes observed with Ringer solution in our work. (Flow will be about halved when the temperature is allowed to fall from 37° C. to 0° C.) It is well known however that there is a much wider range of changes in flow in the intact rabbit ear with changes in temperature. These changes must be neuromuscular in origin, but we should not ignore the fact that these latter effects will be superimposed upon the viscosity effect.

The method of preparation of the ear that we have employed has eliminated the possibility of any central or spinal reflex effects, and so any such effects would have to be produced by either the influence of temperature on the nerve endings in the ear, producing local reflexes, or on the smooth muscles in the vascular walls. That all changes in flow can be accounted for by viscosity changes indicates to us that there is no such intrinsic response of the smooth muscle in the ear. Moreover, removing the reactivity of the smooth muscle by cyanide does not change the effect of temperature.

Neither the rapid oscillations in flow nor the slower "hunting reaction" were observed in the isolated rabbit ear. We would not have expected to have found the more rapid oscillations since, as has been pointed out, these appear to be at least partly central in origin. On the other hand we might have expected to have found the "hunting reaction" since, as mentioned above, the work of Lewis, Greenfield, and Shepherd have indicated the probability of a local origin. It is of interest that we were unable to find the phenomena in the isolated ear.

The absence of any effect of temperature on the residual "critical closing pressure" strengthens our belief expressed in an earlier paper (24) that this pressure is due to interfacial tension between the vascular wall and the fluid contained therein.

V. Conclusions

Our experiments have indicated to us that, in the isolated perfused rabbit ear, changes in flow produced by changes in temperature can be completely explained on the basis of changes in viscosity of the perfusion solution. The resistance to flow increases by two and one-half times when the temperature falls from 40° C. to 0° C. Freeman and associates have reported the changes in flow with changes in temperature in the sympathectomized hand (11). It may be that a substantial part of the changes which they observed in cases of recent sympathectomy could be explained on the basis of purely viscous effects. Six months after operation, the changes with temperature are too great to be explained by viscous effects alone.

The isolated perfused rabbit ear in our experiments shows no intrinsic responses to changes in temperature in so far as could be detected by flow measurements. No sign of the "hunting reaction" to cold was seen when the inflowing solution was either at the same or at a higher temperature than the tissues of the ear.

The low "residual" critical closing pressure in the isolated rabbit ear, which is without vasomotor tone, is not significantly affected by temperature.

VI. Summary

1. In the isolated perfused rabbit ear, changes in flow produced by changes in temperature can be completely explained on the basis of changes in viscosity of the perfusion solution, when the perfusion pressure at the artery is kept constant.

2. The magnitude of the "critical closing pressure" in the isolated perfused rabbit ear does not appear to be a function of temperature.

Acknowledgments

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HEPARINASE

II. DISTRIBUTION OF ENZYME IN VARIOUS TISSUES AND ITS ACTION ON NATURAL HEPARINS AND CERTAIN SYNTHETIC ANTICOAGULANTS[1]

By L. B. Jaques and Eve Keeri-Szanto

Abstract

The method developed by Monkhouse and Jaques (1950) for the determination of heparin in plasma has been applied to the study of the enzyme, heparinase, first described in 1940 by Jaques. The enzyme can be prepared from rabbit liver by glycerol extraction and precipitation with half-saturated ammonium sulphate. It has a pH optimum 6.0–6.5. Extracts from rabbit kidney, lung, heart, aorta, muscle, intestine, spleen, testis, brain, and blood and beef liver, lung, and blood were inactive. The metachromatic and antithrombin properties of heparin decrease in a parallel fashion under the action of the enzyme. The enzyme also acts on sheep heparin and the synthetic polysaccharide, treburon.

Introduction

In 1940, Jaques (2) reported the preparation of an enzyme from rabbit liver which inactivated heparin. However, the only method of following the action of the enzyme was by biological assay and it appears that because of the laborious nature of these tests, no further study of this enzyme has been attempted. Recently, Monkhouse and Jaques (9) have described a simplified procedure for the separation of heparin from protein in plasma to allow its measurement by chemical tests. In the present study this method has been applied to the separation and measurement of heparin in enzyme digests.

Materials and Methods

The heparinase was prepared, as described by Jaques (2), from rabbit livers by extracting with glycerol, dialyzing, precipitating with ammonium sulphate, and redialyzing.

Instead of mincing the livers for the extraction of heparinase, they were put through a Waring Blendor for three minutes, which resulted in a more homogenous suspension and a more active extract. In later experiments toluene was used as bacteriostatic instead of tricresol, since the latter repeatedly precipitated the proteins in the extract.

One milliliter of the final heparinase preparation (corresponding to 5 gm. liver tissue) was diluted with 8 ml. of physiological saline and the hydrogen-ion concentration of the mixture was adjusted, using the Beckman pH meter and glass electrode, to pH 6.0–6.15 with 0.1 *N* hydrochloric acid. The proteins present provided effective buffering. One milliliter of heparin solution (containing 100 units of commercial beef heparin, Connaught Medical Laboratories, Toronto) was added and immediately after mixing a 1 ml. aliquot was removed

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and pipetted into 1 ml. of 80% phenol (800 ml. phenol liquefied by warming and diluted to 1 liter with distilled water) to form the sample at zero time. The enzyme–substrate mixture was incubated at 37° C. and further 1 ml. samples were removed at intervals and treated similarly. After mixing well with the phenol each sample was treated in the same way as the plasma samples in the Monkhouse and Jaques (9) procedure. The mixtures were allowed to stand overnight. The aqueous layer was then removed and washed once with ether equilibrated with water. The remaining ether was removed by warming in a water bath at 65° C. for one or two minutes and the heparin content of the aqueous layer was then determined.

Since Jaques, Charles, and Waters (8) have shown that heparin from different species gives quite different assay values in different tests, all assays were done by two methods. The heparin was measured for its metachromatic activity with Azure A in the Lovibond Tintometer by the method of Jaques, Ricker, and Bruce-Mitford (7), using the glass cell described by Jaques, Monkhouse, and Stewart (6). The antithrombin activity was measured against Parke Davis topical thrombin by the method of Jaques and Charles (3) with oxalated dog or beef blood, using a 0.025 unit end point. In the experiments with uroheparin as substrate, owing to colored impurities in the uroheparin, the Lovibond Tintometer could not be used and the metachromatic activity was measured in a Beckman Spectrophotometer Model DU, from the ratio of light absorption at 500 mμ to 540mμ. The details of this method, which has recently been developed in this laboratory, will be published separately (4).

Heparin concentrations have been expressed in all cases in units. The metachromatic unit and antithrombin unit are the metachromatic and antithrombin activities respectively of 1/117 mgm. of our laboratory standard heparin, which was found by direct comparison with a sample of the international standard of heparin to contain 117 international units per mgm., as judged by antithrombin potency.

Experimental

pH Activity Curve

Under the conditions given by Jaques (1), 1 ml. of the enzyme preparation at pH 6.0 was consistently found to destroy 35% of the added heparin in 24 hr. by the tests used. No further change of activity was found in the next 24 hr. These results are in excellent accord with the findings reported in Jaques' original publication, based on biological assay. This indicated that the method could be used to study heparinase activity. The effect of pH on enzyme activity was then studied by preparing mixtures of enzyme and heparin at various pH levels. The results are shown in Table I. The control consisted of the same quantities of enzyme and heparin incubated separately for 24 hr., then mixed and precipitated with phenol immediately. It can be seen that assay values for control and zero sample were in good agreement, assuring that removal of heparin activity by nonenzymatic action did not

TABLE I

EFFECT OF pH ON ACTIVITY OF HEPARINASE

pH	Units of heparin in reaction mixture						Decrease in activity, %
	Control		0 time		24 hr.		
	M.A.	A.T.	M.A.	A.T.	M.A.	A.T.	
4.0	9.0	9.05	9.2	9.2	8.2	8.2	11
5.0	9.1	9.2	9.2	9.2	6.9	7.0	25
6.0	9.0	9.1	9.0	9.0	6.2	6.0	34
6.5	9.0	9.0	9.0	9.0	6.0	6.0	34
7.0	9.0	9.0	9.0	9.0	8.4	8.4	7
6.1	9.1	9.0	9.1	9.1	6.2	6.0	34
6.1 PO_4 buffer	9.0	9.1	9.0	9.1	6.2	6.2	32

NOTE: M.A. = *Metachromatic activity.*
A.T. = *Antithrombin activity.*

occur. Maximal enzymatic activity was observed in the samples at pH 6.0 and pH 6.5 with very little activity at pH 4 and pH 7. This is in complete agreement with the earlier results of Jaques. In a separate experiment the effect of the presence of phosphate buffer was tested. This did not appear to affect the activity of the enzyme. Of considerable importance is the observation, shown in Table I, that the disappearance of metachromatic activity paralleled the loss of antithrombin activity.

Properties of the Enzyme

Glycerol appears to be necessary for the initial extraction of the enzyme from the liver. Extraction with distilled water and with saline gave definitely lower activity. The purified enzyme preparation was reasonably stable. Kept at 37° C. and pH 6.1 for 48 hr., it lost only 13% of its initial activity. Fractionation with ammonium sulphate was attempted, but no significant purification was achieved as relatively little precipitate was obtained at 25 and 33% saturation with ammonium sulphate.

The distribution of the enzyme was studied (Table II). Tissues were extracted and the extracts treated as for rabbit liver. The lungs, heart, and aorta were extracted together. The intestine included both small and large intestine and the contents were removed by washing before extracting. The hair was removed from a sample of skin by clipping and after being cut in small pieces this was extracted with glycerol. This preparation failed to give a precipitate with ammonium sulphate, so it was not possible to obtain values for skin. The blood was added directly to the glycerol which lyzed and dissolved the cells. The spleen, testis, and brain were triturated in a mortar instead of the Waring Blendor. It was found necessary to comminute the muscle in a domestic meat grinder before using the Blendor. In view of the nature of the test, the results have only qualitative value and cannot be given

TABLE II

DISTRIBUTION OF ENZYME

1 cc. (100 units) heparin; 1 cc. *M*/10 acetate–phosphate buffer, pH 6.1; + enzyme extract equivalent to 5 gm. of tissue; + saline. Total vol. = 10 cc.

Tissue	Heparin, units/ml.				% Heparin destroyed	Enzyme activity
	Zero sample		48 hr.			
	M.A.	A.T.	M.A.	A.T.		
Rabbit						
Liver	10.9	9.2	6.5	6.5	35	+
Kidney	9.0	15.2	9.8	14.2	Nil	—
Lung–heart–aorta	12.2	14.2	12.0	—	Nil	—
Muscle	7.4	9.3	5.0	16.2	?	?
Intestine	12.4	10.0	12.6	15.6	Nil	—
Spleen	9.5	11.9	14.3	20.6	Nil	—
Testis	4.2	2.4	4.8	10.8	Nil	—
Brain	13.8	15.0	14.5	22.7	Nil	—
Blood	5.7	—	11.0	15.0	Nil	—
Beef						
Liver	6.6	22.1	11.4	10.6	Nil	?
Lung	8.4	16.2	9.0	19.6	Nil	—
Blood	6.4	13.1	6.5	11.2	Nil	—

NOTE: M.A. = *Metachromatic activity.*
A.T. = *Antithrombin activity.*

any quantitative significance. Of the various tissues extracted, only that from rabbit liver showed significant enzyme activity.

Some of the mixtures show greater metachromatic activity than can be accounted for by the heparin added. As this was also shown on the antithrombin assays, it probably represents heparin present in the tissue extract. In the case of kidney, spleen, and beef lung the amount of this recovered by the phenol method appeared to increase on incubation, a well-known phenomenon in extracting heparin from tissue. On the other hand in the case of the extracts from rabbit blood, beef liver, beef blood, and testis, the zero samples only gave 60% of the theoretical metachromatic activity. This was probably due to substances present in the extract which interfered with the color reaction, since with the first two, full metachromatic activity was recovered after 48 hr. incubation. Irregular results were also obtained with the antithrombin assays. This also was probably due to accompanying impurities, which apparently caused the antithrombin assay to read too high. Technical difficulties of this type were observed and discussed previously when the biological assay was used. As a result of these discrepancies, the results obtained with extracts of rabbit muscle and beef liver appear equivocal. However, for the beef liver extract, the heparin in the mixture after incubation was the same as blank controls by both methods of assay, indicating that no enzymic inactivation had occurred, while for the muscle extract after incubation, the antithrombin activity reading was as much greater than that of the

blank, as the metachromatic activity was lower, which suggests that the extract does not contain enzymatic activity comparable to that obtained from rabbit liver. Extracts from other tissues definitely did not destroy heparin under these conditions.

Other Substrates

Jaques *et al.* (5) have obtained, on a number of occasions, an excretion product of heparin from urine. This substance, uroheparin, possesses metachromatic activity but little antithrombin activity. Mr. S. W. Levy kindly supplied several samples of partially purified uroheparin. A recent synthetic sulphonated polysaccharide, treburon, was kindly supplied by Hoffman-La Roche. Finally, a sample of sheep heparin, prepared by one of us (L.B.J.) in 1939, and a sample of carragin, supplied by Boots Pure Drug Co. Ltd., Nottingham, England, were tested. The results are shown in Table III.

TABLE III

SUBSTRATES FOR HEPARINASE

1 cc. rabbit liver extract + polysaccharide in 10 cc., pH 6.1.

Substrate	Substrate		Metachromatic activity (units/cc.)		Metachromatic activity destroyed in 48 hr., %
	Mgm.	Units	Zero time	48 hr.	
Commercial beef heparin	0.85	100	9.2	5.2	43
Commercial beef heparin	4.25	500	48.0	34.5	28
Sheep heparin	1.0	90	8.6	6.9	19
Uroheparin	10.0	98	8.2	11.9	—
Treburon	1.0	177	16.2	12.9	20
Carragin	1.0	132	12.2	12.2	0
No substrate	—	—	<0.1	<0.1	—

Owing to the relatively low antithrombin activities of most of these substrates, this activity could not be assayed and values are reported only for metachromatic activity. For convenience of measurement, the substrates were used in amounts to give about 100 metachromatic units, which required about 1 mgm. each of beef heparin, treburon, and carragin and 10 mgm. of uroheparin. It can be seen with beef heparin that increasing the amount of substrate increased the absolute amount of substrate destroyed but resulted in a slight decrease in the percentage of substrate destroyed. The enzyme attacked sheep heparin and also treburon, a synthetic anticoagulant. On the other hand carragin, a sulphated polysaccharide from seaweed, was not attacked. Carragin is a galactan while treburon is a sulphonated polygalacturonic acid methyl ester methyl glycoside. Results on uroheparin were equivocal. On one occasion, there was a decrease in metachromatic activity, but, in two experiments, there appeared to be an increase in metachromatic activity. The significance of this finding is not apparent at present and requires further study.

Discussion

The fact that heparin disappears rapidly from the circulation has long been known but the nature of this process has been obscure. The existence of an enzyme which destroyed heparin was announced in 1940. However, the necessity for biological assays prevented any further development. The results reported above demonstrate that the method of Monkhouse and Jaques for the separation of heparin from plasma proteins is equally satisfactory for enzyme digests. It is also highly significant that using this new and entirely different approach, the results obtained are in complete agreement with those obtained in 1940.

In all experiments, the decreases in metachromatic and antithrombin activity were identical. This probably means that the metachromatic property of heparin is affected by heparinase. An alternative explanation is that the product of heparinase action is not recoverable by the phenol procedure, because of solubility in phenol, etc. This experimental observation is of great practical interest, for it makes it possible to follow heparinase action by relatively simple colorimetric methods. If it also means that heparinase affects the metachromatic property of heparin, it is also of considerable theoretical significance, since it both throws light on the physiological significance of heparinase and is contrary to what might have been predicted. With regard to the latter, Jaques, Charles, and Waters (8) showed that when heparins from different species were examined they all had the same specific metachromatic activity, although the specific biological (anticoagulant) activities were markedly different (dog heparin 10 times that of sheep heparin). Mrs. A. G. Ricker, Mr. E. Napke, and Mr. S. Levy, in conjunction with one of us (L.B.J.), have isolated the urinary excretion product of heparin, uroheparin, as the barium salt and found that, while it has a very low biological activity, it still retains almost full metachromatic activity. A priori, therefore, it might be thought that heparinase would leave the metachromatic property of heparin unattacked, which apparently is not the case.

From the evidence cited above, it is evident that uroheparin cannot be the product of the action of heparinase. Mr. E. W. Napke and Mr. S. W. Levy have separately conducted balance studies on injected heparin in man, dog, and rabbit. After subtracting the amount of commercial heparin excreted unchanged, they find that the excretion of uroheparin accounts for less than 15% of the heparin metabolized. Their studies on urine agree with the conclusions of Jaques (1), based on following blood clotting times after the injection of heparin, that urinary excretion is not a factor at blood levels up to two-three units per milliliter. These results can only be explained at the present time on the assumption that the action of heparinase, which gives metabolic products not metachromatically active, is of chief significance in explaining the inactivation of heparin *in vivo*.

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GLUCOSE UTILIZATION IN BLOOD CELLS SURVIVING STORAGE AT −79° C.[1]

By M. C. Blanchaer and C. I. Mayman

Abstract

Human venous blood was mixed with an equal volume of 40% glycerol in 0.85% saline, equilibrated for 1.5 hr. at 5° C. and then frozen at −79° C. in a solid carbon dioxide – alcohol bath. At various intervals specimens were rapidly thawed and the major portion of the glycerol removed from the cells by diffusion during the logarithmic addition of 79 volumes of 1.0% saline. The cumulative erythrocyte loss by hemolysis during this procedure varied from 4 to 15%, depending upon the length of storage at −79° C. The rate of glycolysis in cells surviving one to four weeks at −79° C. was similar to that reported in fresh erythrocytes and greater than that in cells stored for comparable periods at 5° C. in the common blood preservative, acid citrate – dextrose.

Introduction

Smith (14) and Sloviter (12) have recently reported that whole blood mixed with an equal volume of saline containing 30% glycerol may be frozen at −79° C., thawed, and then freed of glycerol with little hemolysis. This report was followed by evidence (13) that rabbit erythrocytes preserved under these conditions for as long as 42 days are viable when re-injected into the circulation. More recently Mollison and Sloviter (8) have shown that human erythrocytes kept in glycerol at −79° for three hours survive transfusion as well as untreated cells.

It is now generally agreed (2, 6, 11) that the ability of red cells preserved in the conventional citrate solutions to remain in the circulation after transfusion is related to their continued utilization of glucose and is probably dependent upon the maintenance of an adequate cellular adenosine triphosphate level (2). The present work was undertaken to determine whether erythrocytes preserved in glycerol retain sufficient glycolytic activity to warrant *in vivo* survival tests on cells stored in this manner for periods longer than those studied by previous workers.

Experimental

In preliminary experiments it was found that the addition of blood to 40% glycero-saline produced less hemolysis than when 20% or 30% glycero-saline was used as recommended by Sloviter. Therefore, in the experiments reported here, fresh defibrinated human blood was mixed with an equal volume of a solution containing 40 gm. glycerol ("Glycerin", A. R., Mallinckrodt) and 0.85 gm. sodium chloride per 100 ml. Samples of the blood–glycero-saline mixture, varying from 6 to 10 ml., were allowed to stand 1.5 hr. at 5° C. in rubber-stoppered 18 × 150 mm. Pyrex test tubes sealed with parafilm and then placed in a solid carbon dioxide–alcohol bath at −79° C. At various intervals

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noted below, samples were removed from the bath and rapidly thawed by placing them in a water bath at 37° C. for exactly three minutes. Most of the glycerol in the cells was then removed by diffusion during the logarithmic addition of 79 volumes of 1% saline at room temperature to 2 ml. of the thawed suspension with thorough mixing according to the equation:

$$\text{Log } V_T = \log V_I + 0.0434\ T$$

V_I being the initial volume of the thawed blood–glycero-saline mixture in milliliters and V_T the total volume of the mixture of 1% saline plus blood–glycero-saline at T minutes. The saline was added from a burette according to a schedule which indicated the expected residual saline in the burette at any given time and also the volume of saline to be added during successive one minute intervals. This procedure, which to our knowledge has not been used for this purpose previously, is more rapid than the dialysis method of Sloviter since a constant high glycerol gradient from the cells to the ambient fluid is maintained by the progressive increase in the rate of saline addition. Cells subjected to this procedure were calculated to contain a final concentration of 0.25% glycerol, on the assumption that the extra- and intracellular concentration of glycerol reached equilibrium shortly after the addition of saline was completed. Following centrifugation at 850 × g for 15 min. to remove the cells, the hemoglobin concentration in the supernatant fluid was estimated (1) and used to calculate the cumulative hemolytic effect of the addition of glycerol to blood followed by freezing, thawing, and removal of the major portion of the intracellular glycerol by dilution. Blood from three healthy young men was used in these experiments.

It may be seen in Table I that mixing blood with glycero-saline and subsequently removing most of the glycerol *without* freezing resulted in a 4 to 5%

TABLE I

HEMOLYSIS IN BLOOD MIXED WITH GLYCERO-SALINE

Mixtures: equal volumes of	Time at −79° C.	% Red cells lost by hemolysis		
		Experiment		
		1	2	3
Blood + 1% saline	0	0	—	1.2
Blood + 40% glycero-saline	0	—	4.8	4.1
" " "	17 hr.	—	8.3	—
" " "	7.5 days	14.5	9.9	11.2
" " "	14 days	14.0	14.6	13.8
" " "	28 days	—	—	12.0

loss of the cells by hemolysis. Freezing at −79° C. approximately doubled the loss but little additional hemolysis occurred during the second week.

The glycolytic capacity of the thawed red cells was examined after they had been separated by centrifugation following the logarithmic dilution procedure. Sufficient of the supernatant solution was removed to leave approximately a 3 : 1 ratio of supernatant to cells before the latter were resuspended. The following materials were then added to the final concentrations indicated: 200–400 mgm. % glucose, 3.8 mgm. % dihydrostreptomycin sulphate, and 1900 units potassium penicillin per 100 ml. The antibiotics suppressed bacterial growth but were shown in preliminary experiments to have no effect on the rate of glycolysis. Glucose utilization by the suspension was determined by triplicate glucose analyses at the beginning and end of a 23 to 25 hr. incubation period at 37° C. The method used was that of Nelson (10) with the protein precipitating reagents of Somogyi (15). The average deviation from the mean value in the sets of triplicate analyses was 1.41% (S.D. ± 1.56). Glucose utilization was expressed in milligrams glucose consumed per hour by the quantity of cells containing 1.0 gm. hemoglobin since this unit is independent of such changes in cell size as may be produced by freezing and thawing.

It may be seen in Table II that the glycolytic activity of cells prepared in this manner was high but apparently decreased during storage at −79° C.

TABLE II

GLUCOSE UTILIZATION BY RED CELLS AFTER VARIOUS TREATMENTS

Mixtures: equal volumes of	Time at −79° C.	Hemoglobin concentration, gm. %	Glucose in red cell suspensions					
			Mgm., %		Mgm. utilized/hr./gm. hemoglobin			
					Experiment			
			Initial	Final	1	2	3*a*	3*b*
Blood + 1% saline	0	8.6	272	186				0.41
Blood + 40% glycero-saline	0	8.0	177	53	0.63			
		7.1	178	97		0.49		
		7.5	383	279			0.59	
		8.7	265	198				0.32
" " "	17 hr.	6.7	177	104		0.47		
" " "	7.5 days	7.4	174	102	0.41			
		6.7	162	118		0.29		
		8.0	256	185			0.36	
		7.8	312	226				0.44
" " "	14 days	5.4	146	103		0.33		
		6.7	245	182			0.40	
		6.7	209	159				0.31
" " "	28 days	7.5	277	204			0.41	
		7.4	283	220				0.36

In order to examine the effect of removing most of the remaining (0.25%) intracellular glycerol, a portion of the cells from each thawed sample in the third experiment was equilibrated with an additional 30 volumes of saline following the logarithmic dilution procedure.

The glycolytic activity of these cells is shown in Column *3b* of Table II with the corresponding data on the control specimens in Column *3a*. Removal of most of the residual glycerol apparently depressed glycolysis in three of the four samples in this experiment. However, using the hemoglobin concentration as a measure of the number of cells in the suspensions, it was found that the rate of glucose utilization was directly related to the amount of glucose available per cell at the beginning of the 24 hr. incubation period (Fig. 1).

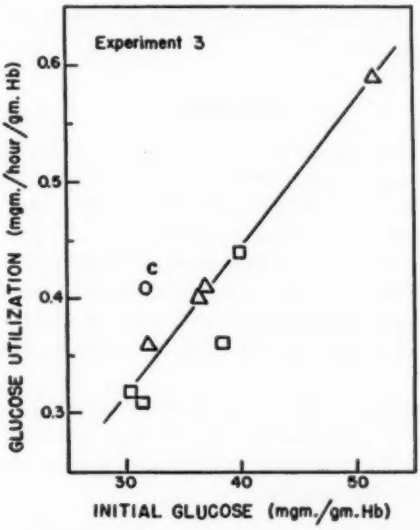

FIG. 1. The relationship between the rate of glucose utilization and the amount of glucose initially available per unit of cells. "C" refers to the saline control, △ to cells containing 0.25% glycerol, □ to washed cells.

Since the removal of the residual glycerol did not alter this relationship, it would appear that the lower rate of glycolysis, of the samples in Column *3b*, Table II, was an artefact arising from accidental differences in the initial glucose concentrations. In view of these findings, it is advisable to withhold judgment at present on the apparent decrease of glucose utilization during storage at −79° C. since such changes in Experiments 2 and 3 may also have been related to differences in initial glucose concentration. It is of interest, however, that in *fresh* blood considerable variations in glucose concentration have little effect on the rate of glycolysis (3, 4).

For purposes of comparison, the glucose utilization was also studied in the washed cells of blood which has been preserved in acid citrate – dextrose

(5, 6, 11). Two sterile samples of blood which had been kept at 5° C. for 27 and 33 days in this preservative were each mixed with 60 volumes of 1% saline and allowed to stand for one-half hour to permit the greater portion of the excess glucose of the preservative to diffuse from the cells. The cells were then collected by centrifugation and mixed with glucose and antibiotics as in the previous experiments. It was found that the two suspensions consumed 0.171 and 0.137 mgm. glucose per hr. per gm. hemoglobin respectively when incubated for 24 hr. at 37° C. A comparison of these results with those in Table II indicates that glucose utilization was considerably more active in the washed cells after preservation at −79° C. than in those kept at 5° C. in acid citrate – dextrose for similar periods. The glycolytic rate of the frozen cells also compares favorably with that of fresh blood which may be calculated from the values in the literature (3, 7, 9) to fall between 0.40 and 0.50 mgm. per hr. per gm. hemoglobin at 37° C.

Discussion

It should be noted that the criterion of continuing glycolytic activity is of value mainly as an exclusion test. While it is probable that stored cells which fail to glycolyze will not survive in the circulation, the preservation of glycolytic capacity is not sufficient in itself to assure survival after transfusion (2). Caution is particularly necessary in interpreting the present results in view of the unexpected dependence of the rate of glycolysis on glucose concentration. However, in spite of these reservations, it is felt that the findings are sufficiently encouraging to warrant further *in vivo* survival studies on blood preserved in glycerol at −79° C.

Although some modification of our methods would be necessary to prepare 500-ml. lots of blood for transfusion, it is felt that the logarithmic dilution procedure is more readily adaptable to mechanized large scale preparations than are methods involving dialysis.

Acknowledgments

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